Molecular and genetic strategies to reduce the susceptibility of wine grapes to fungal pathogens



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CSP 0903**

Principal Investigators: **Dr Ian Dry & Dr Mark Thomas**

Research Organisation: **CSIRO Plant Industry**

Date: **31 January 2014**

Project Title: **Molecular and genetic strategies to reduce the susceptibility of wine grapes to fungal pathogen**

GWRDC Project Number: CSP 0903

Period Report Covers: 1/07/2009 - 31/12/2013

Author Details: Dr Ian Dry & Dr Mark Thomas
CSIRO Plant Industry

Postal address: PO Box 350, Glen Osmond, SA 5064, Australia

Phone: 08 83038632
Fax: 08 83038601
Email: ian.dry@csiro.au

Date report completed: 31/01/2014

Publisher: CSIRO Plant Industry

Copyright and Disclaimer:
© 2014 CSIRO. To the extent permitted by law, all rights are reserved and no part of this publication covered by copyright may be reproduced or copied in any form or by any means except with the written permission of CSIRO.

Important Disclaimer:
CSIRO advises that the information contained in this publication comprises general statements based on scientific research. The reader is advised and needs to be aware that such information may be incomplete or unable to be used in any specific situation. No reliance or actions must therefore be made on that information without seeking prior expert professional, scientific and technical advice. To the extent permitted by law, CSIRO (including its employees and consultants) exclude all liability to any person for any consequences, including but not limited to all losses, damages, costs, expenses and any other compensation, arising directly or indirectly from using this publication (in part or in whole) and any information or material contained in it.

Table of Contents

1. Abstract 5

2. Executive summary 7

3. Background 9

4. Project Aims 11

5. Functional characterisation of the *MrRUN1 & MrRPV1* resistance genes 13

6. Evaluation of resistance of *MrRUN1* & *MrRPV1* transgenics against powdery and downy mildew isolates from Europe and North America 29

7. The search for new resistance genes to grapevine powdery mildew and downy mildew and the development of new transgenic strategies to enable pyramiding with *MrRUN1* & *MrRPV1* 35

8. The role of *VvMLO* genes in powdery mildew susceptibility 49

9. Identification of putative *Erysiphe necator* effectors secreted during infection 57

10. Outcome / Conclusions 59

11. Recommendations 61

Appendix 1: Communication 63

Appendix 2: Intellectual Property 65

Appendix 3: References 67

Appendix 4: Staff 71

Appendix 5: Supplementary Data 73

Appendix 6: Budget reconciliation 75

This page intentionally left blank

1. Abstract

Functional characterisation of transgenic winegrape varieties containing the *MrRUN1* powdery mildew resistance gene and the *MrRPV1* downy mildew gene from *M. rotundifolia* demonstrated that these genes confer strong resistance to mildew isolates from Australia, Europe and North America. However, a powdery mildew isolate from SE USA was identified which can break *MrRUN1* resistance. New sources of genetic resistance to powdery and downy mildew from other North American and Chinese Vitis species were identified which could ultimately be combined with *MrRUN1* and *MrRPV1,* in the same grapevine variety, to enhance the durability of this resistance in the vineyard.

This page intentionally left blank

2. Executive summary

Powdery mildew caused by the fungus *Erysiphe necator* (syn. *Uncinula necator*) and downy mildew caused by the oomycete *Plasmopora viticola* are the two most economically important grapevine diseases worldwide. The cultivated winegrape species*, Vitis vinifera*, has little or no genetic resistance to these pathogens. As a result, control of these diseases is entirely dependent on the widespread application of fungicides. Schofield and Morison (2010) estimated that costs of powdery mildew and downy mildew disease management and yield losses to the Australian Wine Industry are of the order of 76 and 63 million AUD per year, respectively. There is also increasing world-wide pressure to reduce agrochemical use for the control of plant pathogens on crops grown for human consumption.

This project builds on and extends the research initiated in the previous GWRDC project CSP0604 aimed at developing novel molecular and genetic strategies to reduce the susceptibility of winegrapes to powdery and downy mildew and reduce the industry's dependence on fungicide application. The current project has completed the functional characterisation of transgenic premium winegrape varieties containing the *MrRUN1* powdery mildew resistance gene and the *MrRPV1* downy mildew gene from the wild North American grapevine *M. rotundifolia.* Our results demonstrate that the *MrRUN1* gene confers complete resistance to powdery mildew isolates in Australia, Europe and north-eastern USA, but is broken by isolates collected from south-eastern USA from where Muscadine vines originate. The *MrRPV1* gene from *M. rotundifolia* confers strong resistance to downy mildew isolates in Australia, Europe and North America. The *MrRUN1/MrRPV1* locus has also been successfully introgressed into premium winegrape varieties by marker-assisted selection as part of GWRDC project CSP 0904 "*Advanced grapevine genetics for varietal improvement".* Our analysis indicates that these vines provide good resistance to powdery and downy mildew in unsprayed blocks in the Barossa valley, but further evaluation will be required in different vineyard sites throughout Australia. Interestingly some grape genotypes appear to have a minor suppressive effect on resistance conferred by *MrRUN1 and MrRPV1,* but this is unlikely to reduce their effectiveness under growing conditions in most Australian vineyards.

The introduction of the *MrRUN1* and *MrRPV1* genes into new winegrape cultivars either through grapevine transformation or conventional breeding offers the potential to dramatically reduce fungicide applications in Australian vineyards. However, to date, there are few examples of single dominant *R* genes in other crops that have proven to be durable in the field. In order to improve the durability of genetic resistance in the field, the second major aim of this project was to identify other unique sources of powdery and downy mildew resistance to complement the action of *MrRUN1* and *MrRPV1*. This "pyramiding" of different resistance genes, within the same plant, has been shown to be important in maintaining durable field resistance in other crops. New sources of genetic resistance to downy mildew were identified in the North American species *V. cinerea* and the Chinese species *V. amurensis,* while new sources *of* genetic resistance to powdery mildew were identified in the Chinese species *V. piasezkii* and *V. romanetii* (*REN4*). In collaboration with researchers at the USDA we have obtained evidence to indicate grapevine plants containing the *REN4* powdery mildew resistance locus are resistant to the Muscadine powdery mildew isolates Musc4 and

Musc5 which break *MrRUN1*-mediated resistance, confirming that *REN4* would be an excellent candidate for pyramiding with *MrRUN1* because it would extend the range of isolates to which these vines would be resistant and increase the durability of resistance in the vineyard.

This project has also successfully utilised new Next-Generation sequencing techniques to identify putative effector proteins secreted by grapevine powdery mildew during infection. This opens up the possibility of developing a genetic screen to enable us to track the potential appearance of *MrRUN1*-breaking powdery mildew strains in the vineyard.

Finally, the use of RNAi-silencing technology to suppress the expression of putative powdery mildew "susceptibility" genes in grapevine has proven difficult and its use as an alternative strategy for generating powdery mildew resistant grapevines remains problematic due to potentially deleterious effects of this MLO-silencing strategy on other aspects of grapevine growth and development.

Acknowledgments

The authors of this report would like to thank CSIRO staff Angela Feechan, Angelica Jermakow, Debra McDavid, Nayana Arunasiri, Nicole Kempster, Don Mackenzie, Maria Mrinak and Amanda Walker for valuable contributions to the project. Operating costs ($19k) to fund the Next Generation sequencing component of this project where obtained by Dr Angela Feechan as part of the CSIRO Transformational Biology Initiative.

3. Background

Powdery mildew caused by the fungus *Erysiphe necator* (syn. *Uncinula necator*) and downy mildew caused by the oomycete *Plasmopora viticola* are the two most economically important grapevine diseases worldwide. The cultivated winegrape species*, Vitis vinifera*, has little or no genetic resistance to these pathogens. As a result, control of these diseases is entirely dependent on the widespread application of fungicides. Schofield and Morison (2010) estimated that costs of powdery mildew and downy mildew disease management and yield losses to the Australian Wine Industry are of the order of 76 and 63 million AUD per year, respectively. There is also increasing world-wide pressure to reduce agrochemical use for the control of plant pathogens on crops grown for human consumption. This project builds on and extends research initiated in the previous GWRDC project CSP0604 aimed at developing novel molecular and genetic strategies to reduce the susceptibility of winegrapes to powdery and downy mildew and reduce the industry's dependence on fungicide application.

A major outcome of GWRDC project CSP 0604 was the cloning and functional testing of resistance (*R*) genes at the *MrRUN1* (resistance to *Uncinula necator*) / *MrRPV1* (resistance to *Plasmopora viticola*) locus from the wild North American grapevine *Muscadinia rotundifolia.* This identified two candidate *R* genes that confer resistance to powdery and downy mildew. In this project the functional analysis of these *R* genes has been completed, including an analysis of the resistance response of these genes against mildew isolates from Europe and North America and the performance of the *MrRUN1/MRPV1* locus in different grape genetic backgrounds when planted in the field. Another aspect of research relating to the function of the *MrRUN1 and MrRPV1* resistance genes that was included in this project in the final year, as a result of extra funding being obtained from CSIRO, was a pilot study to investigate the feasibility of using Next Generation Sequencing technology to identify putative fungal effector molecules secreted during infection of powdery mildew-infected grape tissues. Future identification of the secreted effector protein recognised by the MrRUN1 protein would be extremely useful as it would allow us to track the potential appearance of *MrRUN1*-breaking powdery mildew strains in the vineyard.

The introduction of the *MrRUN1* and *MrRPV1* genes into new winegrape cultivars either through grapevine transformation or conventional breeding offers the potential to dramatically reduce fungicide applications in Australian vineyards. However, to date, there are few examples of single dominant *R* genes that have proven to be durable in the field. In order to improve the durability of this genetic resistance in the field, the second major component of this project is to identify other unique sources of powdery and downy mildew resistance to complement the action of *MrRUN1* and *MrRPV1*. This "pyramiding" of different resistance genes, within the same plant, has been shown to be important in maintaining durable field resistance in other crops. Thus, this project will investigate alternative sources of powdery and downy mildew resistance from North American and Chinese Vitis species.

One novel strategy which has been shown to be particularly effective in generating durable, non-race specific resistance to powdery mildew, in other crops, is the down-regulation or silencing of selected members of the plant MLO gene family which have been shown to act as powdery mildew "susceptibility" genes. In the previous GWRDC project CSP 0604, we undertook the cloning and functional characterisation of the seventeen member *VvMLO* gene family in grapevine in an attempt to identify those genes most likely to be involved in

powdery mildew susceptibility. The research identified three genes *VvMLO3*, *VvMLO4* and *VvMLO17* which, based on a number of parameters, are strong candidates for having a role in powdery mildew susceptibility in grapevine. This project will attempt to obtain definitive evidence for this through the generation of transgenic grapevines in which these candidate VvMLO genes have been silenced and evaluating if there has been any effect on powdery mildew susceptibility.

4. Project Aims

1. Complete the functional characterisation of resistance gene candidates located at the MrRUN1/MrRPV1 locus.
2. Evaluate the resistance of transgenic germplasm containing individual R gene candidates to different isolates of powdery and downy mildew.
3. Screen other germplasm including other *M. rotundifolia* cultivars for possible new sources of genetic resistance to powdery mildew and downy mildew other than *MrRUN1* & *MrRPV1*. Identify molecular markers linked to the new resistance locus to be used in MAS for new germplasm containing combinations of resistance genes from different sources.
4. Complete the generation and evaluation of transgenic microvines containing constructs for silencing putative powdery mildew "susceptibility" genes. If successful, similar constructs will be introduced into existing winegrape cultivars for evaluation of vine performance.
5. Subject to the outcome of Objective (4) undertake an Ecotilling project to identify naturally occurring *mlo* mutant alleles in grape germplasm collections for incorporation into new breeding material to generate powdery mildew resistant germplasm.

This page intentionally left blank

5. Functional characterisation of the *MrRUN1 & MrRPV1* resistance genes

Introduction

In the previous GWRDC project CSP 0604, we reported on the cloning and sequencing of all seven major resistance gene analog sequences located at the *MrRUN1/MrRPV1* locus (*MrRGA1*, *MrRGA2*, *MrRGA4*, *MrRGA8*, *MrRGA9* and *MrRGA10*) that had been introgressed from *M. rotundifolia.* We also reported on the generation of over 300 transgenic grapevines containing the individual RGA genes (in collaboration with our colleagues at INRA, Montpellier, France) to determine which of these *RGA*s confer resistance to powdery and/or downy mildew. Although phenotyping of these transgenic vines had not been completed by the end of Project CSP 0604, initial results indicated that: (a) *MrRGA10* is the only major gene responsible for conferring powdery mildew resistance and (b) *MrRGA8* confers strong resistance to downy mildew, but this was based on results in only four transgenic lines. The phenotypic analysis of all transgenic lines has been completed in this current project together with a characterisation of these *R* genes in terms of transcription, cellular targeting of the encoded proteins and the evolutionary relationships of *MrRUN1* and *MrRPV1* with other TIR-NB-LRR genes in *V. vinifera.*

It was also recognized at the end of project CSP0604 that, due to ongoing issues related to the possible deployment of transgenic grapevines in Australian vineyards, we should also investigate the feasibility of introgressing the *MrRUN1/MrRPV1* mildew resistance locus into premium winegrape varieties through conventional hybridization, but use the knowledge gained from sequencing of the *MrRUN1/MrRPV1* locus to develop markers to enable us to employ marker-assisted selection (MAS) to improve the efficiency of conventional breeding. This then formed a major part of GWRDC project CSP 0904 "*Advanced grapevine genetics for varietal improvement"* in which crosses were undertaken between the parental line BC5:3294-R23 containing the *MrRUN1*/*MrRPV1* locus and 17 premium winegrape varieties. The performance of progeny vines generated from these crosses in terms of disease resistance in the field and *in vitro* will also be reported in this section.

Materials and Methods

Plant and fungal material

Potted *Vitis vinifera* cv. Cabernet Sauvignon vines were grown in glasshouses at Waite Campus maintained between 23-25^{0}C. Canes were harvested from selected progeny of BC5:3294-R23 (*MrRUN1/MrRPV1*) x *V. vinifera* crosses at SARDI Nuriootpa and used to establish new potted vines within the CSIRO PI glasshouses to provide material for rechecking powdery and downy mildew resistance.

Erysiphe necator (isolate APC1) culture and inoculations were performed as previously described (Donald *et al*., 2002). *Plasmopora viticola* inoculum was collected from an experimental vineyard on the Waite Campus, Adelaide and maintained on detached leaves of Cabernet Sauvignon. Discs (1 - 1.5 cm diameter, 10 per leaf) were cut from duplicate leaves (nodes 4-7), sprayed with *P. viticola* sporangia suspension in water (~5 x 10^4 sporangia/ml) and incubated in sealed petri dishes at 22 °C under a 16 h light / 8 h dark cycle

for 6 d. Leaves were scored using the OIV 452 descriptor scale. Sporulation was estimated quantitatively using a haemocytometer.

Grapevine RNA extraction, cDNA synthesis and gene expression analysis

Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and DNase treated. cDNA was synthesised using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with the Oligo (dT)20 primer according to the manufacturer's instructions. Transgene expression was measured using qPCR as described previously (Feechan *et al.*, 2008) using *MrRGA*-specific primers. The expression of each target gene was calculated relative to the housekeeping gene elongation factor 1 (*EF1*; GenBank Accession number AF176496) using Rotor-Gene 6.0 software (Corbett Research).

Protein localization studies

RNA extracted from transgenic lines P10-23 and T8-5 and was used to prepare cDNA from which full-length coding regions of *MrRUN1* (*MrRGA10)* and *MrRPV1* (*MrRGA8)* were amplified using primers (Table S1) with added restriction sites: RGA10-ATGF (*Xho*I), RGA10-XBAR (*Xba*I), RGA8-ATGF (*Xho*I) and RGA8-KPNR (*Kpn*I). The amplified products were cloned into pDRIVE (Qiagen), verified by sequencing and then subcloned into pN'GFP (a modified version of vector pART7 (Gleave, 1992) with an added GFP coding region) to generate pMrRUN1:GFP and pMrRPV1:GFP fusion constructs respectively. Plasmid mGFP5-ER was also constructed by subcloning the mGFP5-ER fragment from pBIN35S-mGFP-ER (Haseloff *et al.*, 1997) into pN'GFP. The NLS motif RKRRR present in MrRUN1 and MrRPV1 was mutated to RTSRR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using primers RGA10-NLS-SDMF and RGA10-NLS-SDMR and the template pMrRUN1:GFP to give pMrRUN1nlsmut:GFP and using RGA8-NLS-SDMF and RGA8-NLS-SDMR and the template pMrRPV1:GFP to give pMrRPV1nlsmut:GFP. Onion (*Allium cepa*) epidermal strips were bombarded with GFP fusion constructs as described previously (Selth *et al.*, 2005).

Analysis of MrRGA splice variants

MrRGA transcripts were amplified from cDNA of *MrRGA8* and *MrRGA10* lines and the BC5:3294-R23 line using CB20 designed to the 5' end of exon 1 and CB632 designed to exon 3 to amplify splice variants (Figure 6). *MrRUN1* transcripts were amplified from P10-23 and BC5:3294-R23 cDNA using the primers RGA10-ATGF and RGA10-XBAR. *MrRPV1* transcripts were amplified from T8-6 cDNA and BC5:3294-R23 cDNA in a two stage process: 5'-ends were amplified using primers RGA8-ATGF and CB632 (Table 5, Appendix 5), 3'ends were amplified using primers CB631 (Table 5) and RGA8-KPNR. PCR products were cloned and sequenced and splice junctions verified in at least two independent clones.

Microscopy

Staining of fungal and oomycete structures was performed as described previously (Koch and Slusarenko, 1990). Visualisation and scoring for PCD (at least 100 germinated powdery mildew conidia per replicate) were performed as described previously (Feechan *et al.*, 2011). Onion cells bombarded with GFP-fusion constructs were mounted in 1× PBS and GFP visualized with a Zeiss Axio Imager M1 microscope, GFP filter (470/40 nm excitation, 525/50 nm emission) under 20x magnification. Nuclei were visualized using 1µg/ml of DAPI dissolved in 1 x PBS, using a DAPI filter (365 nm excitation, 445/50 nm emission).

Sequence annotation

The manual annotation of genes present on *V. vinifera* (PN40024) chromosome 12 was carried out using (a) the prediction programs GAZE (Jaillon *et al.*, 2007), EUGENE (Schiex *et al.*, 2001) and JIGSAW (Allen and Salzberg, 2005) (b) the results of sequence comparisons using BLASTX and HMMer and (c) the spliced alignments of available Vitis cDNA and EST transcript sequences. Detailed sequence information of predicted genes is available in the FLAGdb++ database (Derozier *et al.*, 2011). The position of conserved domains within the predicted MrRGA proteins is based on the regions as previously defined (Takken *et al.*, 2006). The TIRplant domain is based on the revised definition as recently proposed (Bernoux *et al.*, 2011). The position of the putative NLS signal in MrRUN1 and MrRPV1 was predicted using PredictNLS available at the PredictProtein server (http://www.predictprotein.org/).

Results

Screening of MrRGA transgenics for resistance against an Australian powdery mildew isolate

Genomic constructs encompassing the complete gene (promoter-coding region-terminator) were introduced into susceptible *V. vinifera* grapevine cultivars including Shiraz, Tempranillo, Portan, Maccabeu and Carignan by Agrobacterium-mediated transformation. Transgenic lines were screened by qPCR at the seedling stage to ensure that *MrRGA* transgene expression levels approximated that observed in the BC5:3294-R23 line. Once vines had reached sufficient maturity, individual leaves were challenged with powdery mildew.

The *MrRGA* transgenic vines were initially screened for resistance to an Australian *E. necator* isolate. A minimum of seven transgenic lines, including at least two different cultivars, were scored for levels of PCD in penetrated epidermal cells, 48 hours post-inoculation (hpi). *MrRGA10* lines had consistently higher levels of PCD in response to *E. necator* penetration than non-transformed controls (Figure 1a). *E. necator*-induced PCD was not significantly increased in any of the transgenic lines expressing *MrRGA1*, *MrRGA2*, *MrRGA9* or *MrRGA11*. However, a small but significant increase in PCD was observed in 1/7 and 2/13 lines expressing *MrRGA4* and *MrRGA8* respectively. The differences in the level of *E. necator*-induced PCD did not appear to be related to differences in the level of *MrRGA* transgene expression (Figure 1b).

Selected *MrRGA10* lines were examined in more detail (Figure 2). Transgenic grapevines expressing *MrRGA10* were found to induce PCD in 67-78% of penetrated epidermal cells, compared to a mean value of 88% in the powdery mildew resistant BC5:3294-R23 line and 11-15% in untransformed lines (Figure 2a). Interestingly, none of the *MrRGA10* transgenic lines examined displayed *E. necator*-induced PCD levels equivalent to that observed in the BC5:3294-R23 line. However, PCD levels were not correlated with *MrRGA10* transcript levels which were found to be higher in all but one transgenic line compared to BC5:3294-R23 (Figure 2b). Trypan blue staining of leaves 7 dpi confirmed that the rapid induction of PCD in penetrated cells of *MrRGA10* vines halts *E. necator* hyphal growth and prevents conidiophore development compared to susceptible cultivars (Figure 2c & d). This demonstrates that *MrRGA10* confers resistance to *E. necator* and has been designated *MrRUN1*.

(b)

	Portan	Tempranillo	Shiraz	Maccabeu	Carignan
MrRGA1	0.6-0.7[1]	n.a.	1.6-11	n.a.	n.a.
MrRGA2	n.a.	1.9-5.5	n.a.	2.1-7.7	n.a.
MrRGA4	2.7-16.7	4.9-16.1	n.a.	n.a.	n.a.
MrRGA8	13.4-14.9	13.6-13.9	4.3-15.7	n.a.	n.a.
MrRGA9	n.a.	0.9-6.2	0.3-1.3	n.a.	n.a.
MrRGA10	1.4-3.5	3.0 - 32.6	0.3-2.2	n.a.	1.2-1.5
MrRGA11	n.a.	0.7- 6.6	0.5-3.0	n.a.	n.a.

[1] *MrRGA* expression levels in transgenic lines relative to the expression level in BC5:3294-R23 (defined as 1.0)

(a)

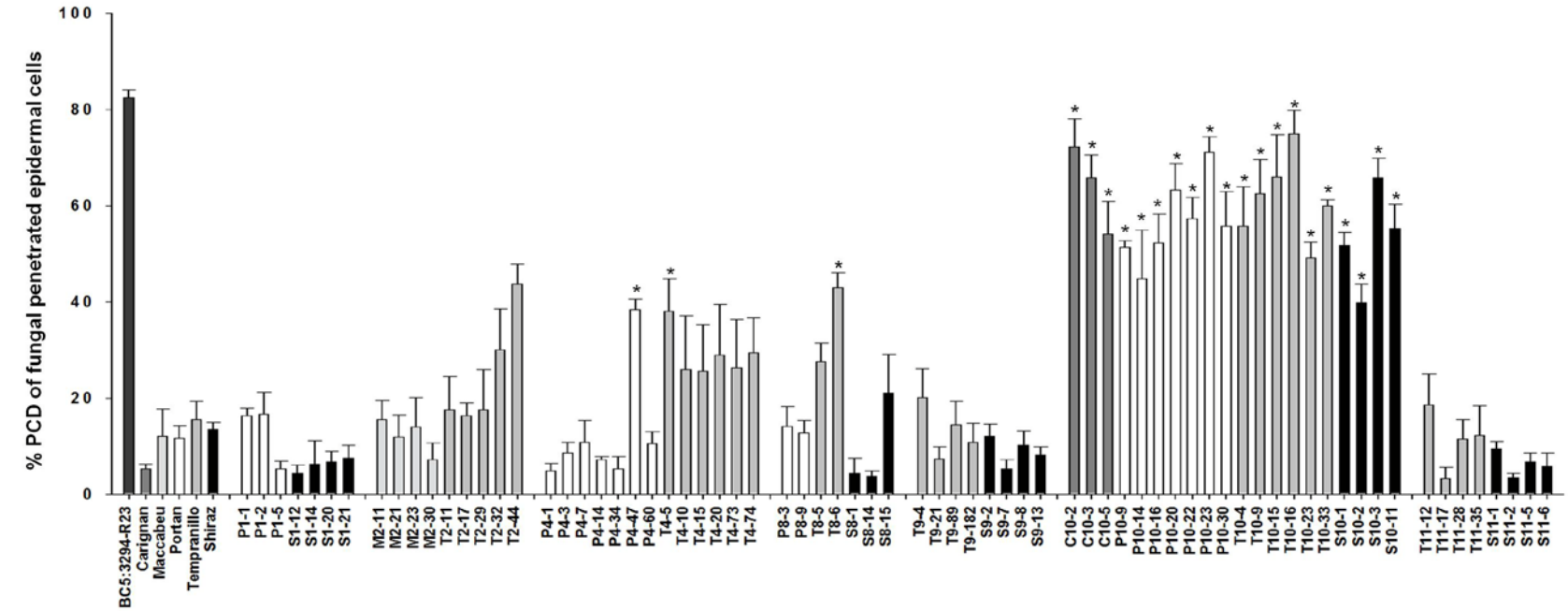


Figure 1. *Screening of transgenic V. vinifera cultivars expressing individual MrRGAs for resistance to an Australian powdery mildew isolate.*
*Detached leaves collected from glasshouse-grown vines were inoculated with the Australian E. necator isolate APC1. Leaves were sampled 48 hpi and scored for the induction of PCD in powdery mildew-penetrated epidermal cells using trypan blue staining. (a) Data is shown for the powdery mildew resistant BC5:3294-R23 line and both untransformed and MrRGA transgenic lines of V. vinifera cultivars Carignan (C), Maccabeu (M), Portan (P), Tempranillo (T) and Shiraz (S). A minimum of seven independent transgenic lines, derived from at least two different cultivars were tested for each MrRGA construct. MrRGA expression levels in transgenic lines, relative to the expression level in BC5:3294-R23 (defined as 1.0) were determined by qPCR on leaves from mature vines. Transcript levels normalised to the expression of the housekeeping gene EF1. Each data point is the mean ± SE (n=2). * indicates that P<0.005.*

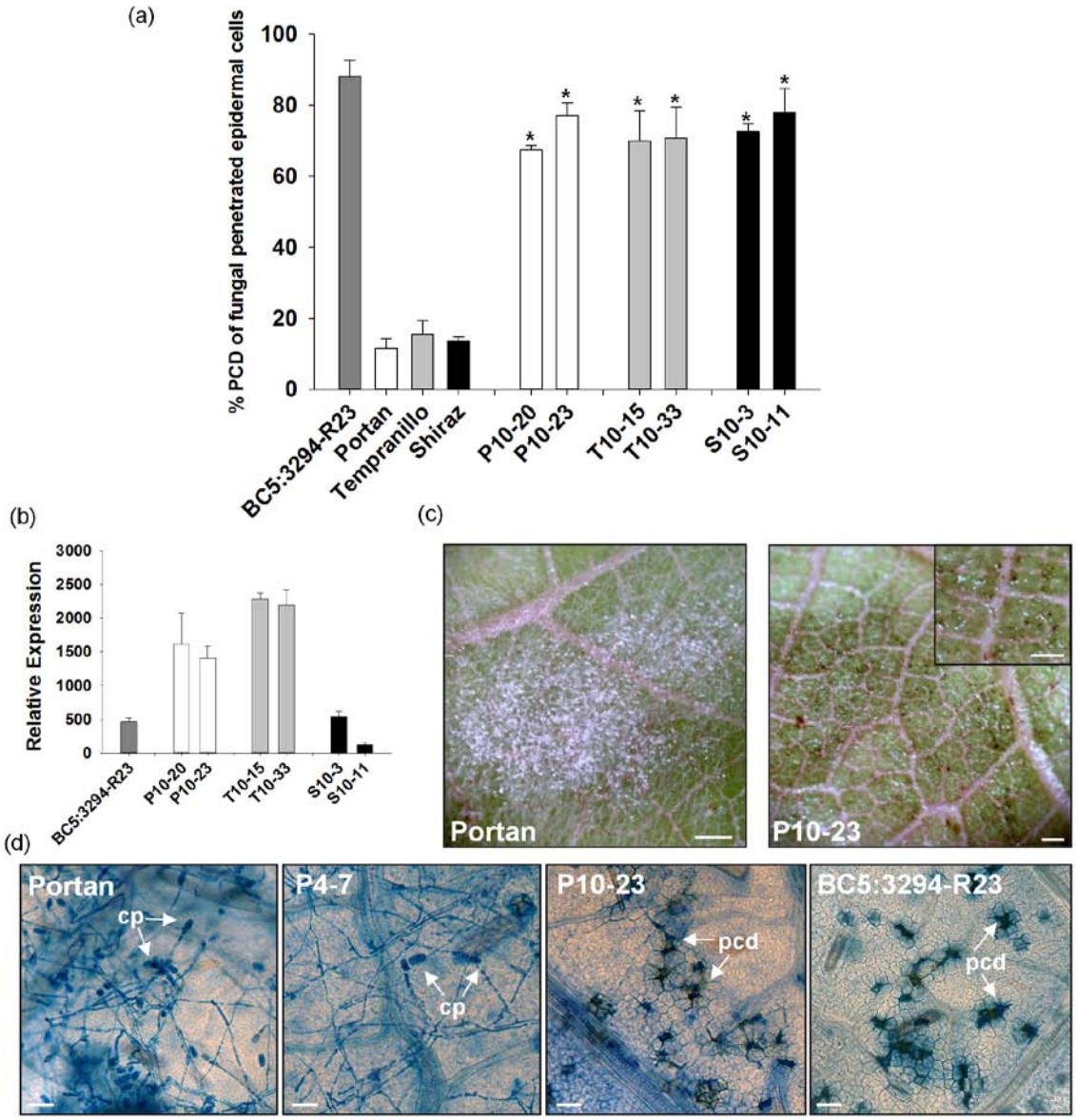


Figure 2. *MrRGA10 (MrRUN1) confers complete resistance to E. necator in transgenic V. vinifera cultivars. (a) Induction of PCD in powdery mildew-penetrated epidermal cells of detached leaves of BC5:3294-R23 (positive control), untransformed V. vinifera cvs. Portan (P), Tempranillo (T) and Shiraz (S) and individual transgenic lines expressing MrRGA10. Leaves were scored 48 hpi and values are the mean ±SE (n=3). * indicates a significant difference from the un-transformed control genotypes Portan, Tempranillo and Shiraz (P<0.005; Student's t-test). (b) Transcript levels of MrRGA10 normalised to the expression of the housekeeping gene EF1. Each data point is the mean ±SE of triplicate testing of two biological replicates. (c) Sporulation on leaves 7 dpi of non-transformed Portan and transgenic line P10-23. Note the absence of conidiophores on the P10-23 leaf and the presence of dark blue/brown spots (epidermal cells which have undergone PCD adjacent to white spores on close-up (inset). (d) Trypan blue staining of powdery mildew inoculated leaves 7 dpi. Lines as above and Portan transgenic line P4-7 expressing MrRGA4. White arrows indicate conidiophores (cp) and epidermal cells which have undergone programmed cell death (pcd) following penetration. Scale bars = 60µm*

Screening of MrRGA transgenics for resistance against an Australian downy mildew isolate

In order to identify the gene(s) responsible for downy mildew resistance, leaf discs of all *MrRGA* transgenic lines were inoculated with an Australian *P. viticola* isolate and scored visually at 6 dpi using the International Organisation of the Vine and Wine (OIV) disease index, which is based on sporulation density and the presence/absence of PCD. An OIV score of 5 or higher is generally considered to indicate a significant level of downy mildew resistance.

Transgenic lines expressing *MrRGA1, MrRGA2*, *MrRGA4, MrRGA9*, *MrRGA10 (MrRUN1)* or *MrRGA11* were found to be as susceptible to *P. viticola* as the non-transformed controls with OIV scores ranging from 1-3 (i.e. dense sporulation, no PCD) (Figure 3). In contrast, the OIV scores of *MrRGA8* transgenic lines of three different *V. vinifera* cultivars were judged to be higher than the downy mildew-resistant BC5:3294-R23 genotype, reflecting the limited sporulation and the presence of PCD observed with these lines.

Selected *MrRGA* lines were investigated in more detail by quantifying sporangia production 6 dpi (Figure 4). *MrRGA8* transgenic lines showed a 92-98% reduction in sporangia production compared to other *MrRGA* lines (including the powdery mildew resistant *MrRGA10* lines) or non-transformed controls (Figure 4a). The reduction in downy mildew sporulation in the *MrRGA8* transgenic lines also exceeded the average reduction (72%) observed in the BC5:3294-R23 genotype which may be related to the much higher levels of *MrRGA8* expression observed in the transgenic lines compared to BC5:3294-R23 (Figure 4b). Trypan blue staining of infected leaf discs 7 dpi revealed profuse *P. viticola* hyphal growth throughout the mesophyll cell layer of susceptible *MrRGA* transgenic lines and non-transformed cultivars whereas in *MrRGA8* lines, hyphal growth and subsequent sporangiophore development was severely restricted by PCD induction (Figure 4c & d). This demonstrates that *MrRGA8* confers resistance to *P. viticola* and has been designated *MrRPV1*.

MrRUN1 and MrRPV1 are targeted to the nucleus

Both MrRUN1 and MrRPV1 contain a predicted nuclear localisation signal (NLS; RKRRR) in the C-terminal domain (Figure 5). Recent studies have indicated that a number of NB-LRR resistance proteins localise to both the cytoplasm and nucleus and that nuclear localisation is essential for disease resistance (Deslandes and Rivas, 2011; Bai *et al.*, 2012). To investigate the cellular localisation of MrRUN1 and MrRPV1, C-terminal GFP fusion constructs were bombarded into onion epidermal cells. MrRUN1:GFP was found to localise predominantly to the nucleus (Figure 5b) whereas MrRPV1:GFP displayed a nucleocytoplasmic location (Figure 5d). Mutation of the conserved NLS in MrRUN1 and MrRPV1 from RKRRR to RTSRR, prevented the nuclear localisation of the GFP fusion proteins thereby suggesting this NLS to be functional (Figure 5c & e)

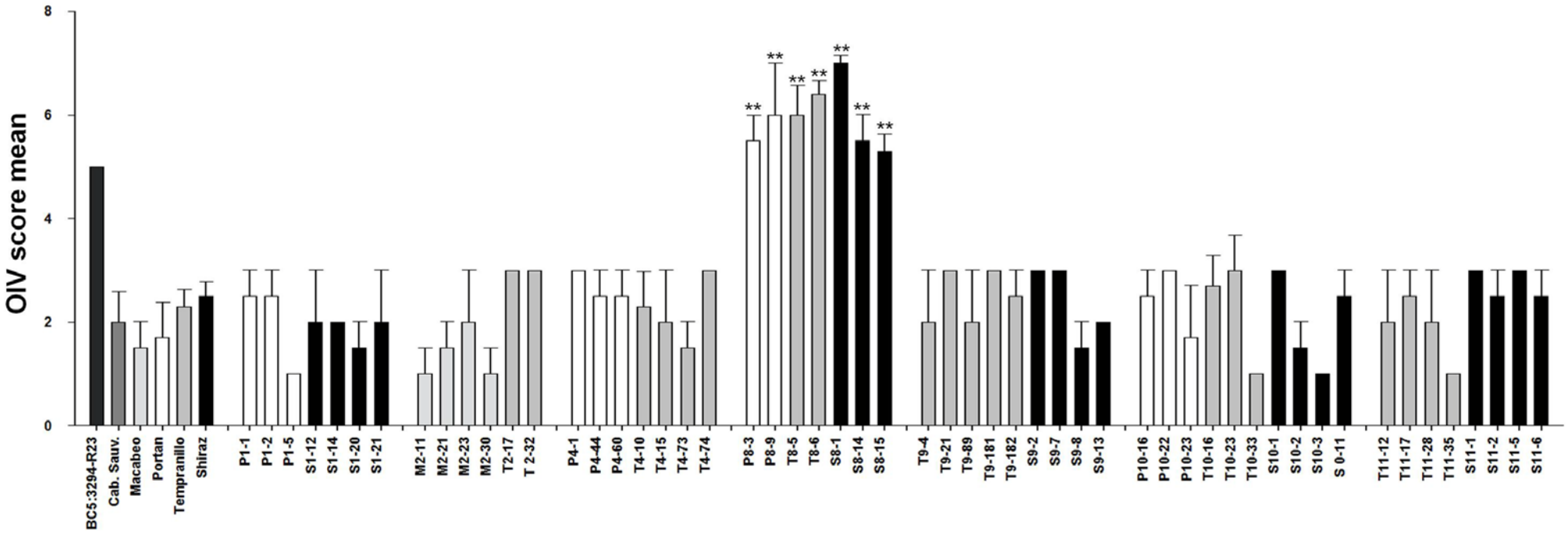


Figure 3. *Screening of transgenic V. vinifera cultivars expressing individual MrRGAs for resistance to an Australian downy mildew isolate.*
*Leaf disc OIV scores of MrRGA transgenic lines of the V. vinifera cultivars Maccabeu (M), Portan (P), Tempranillo (T) and Shiraz (S), untransformed Cabernet Sauvignon and downy mildew-resistant lines BC5:3294-R23 inoculated with P. viticola sporangia of an Australian downy mildew isolate. The OIV disease index assigns scores from 1-9 based on sporulation density and the presence/absence of necrotic lesions: 1 - sporangiophores densely cover the whole disc area, absence of necrosis; 3 - predominant patches of dense sporulation, absence of necrosis; 5 - patches of sparse sporulation equally intermixed with asymptomatic areas, necrotic flecks underneath sporulating areas; 7 - small spots with sparse sporangiophores, necrotic spots or substomatal necrosis; 9 - absence of sporangiophores, necrotic spots or substomatal necrosis. Each data point is the mean ± SE (n=2). ** indicates that P<0.005*.

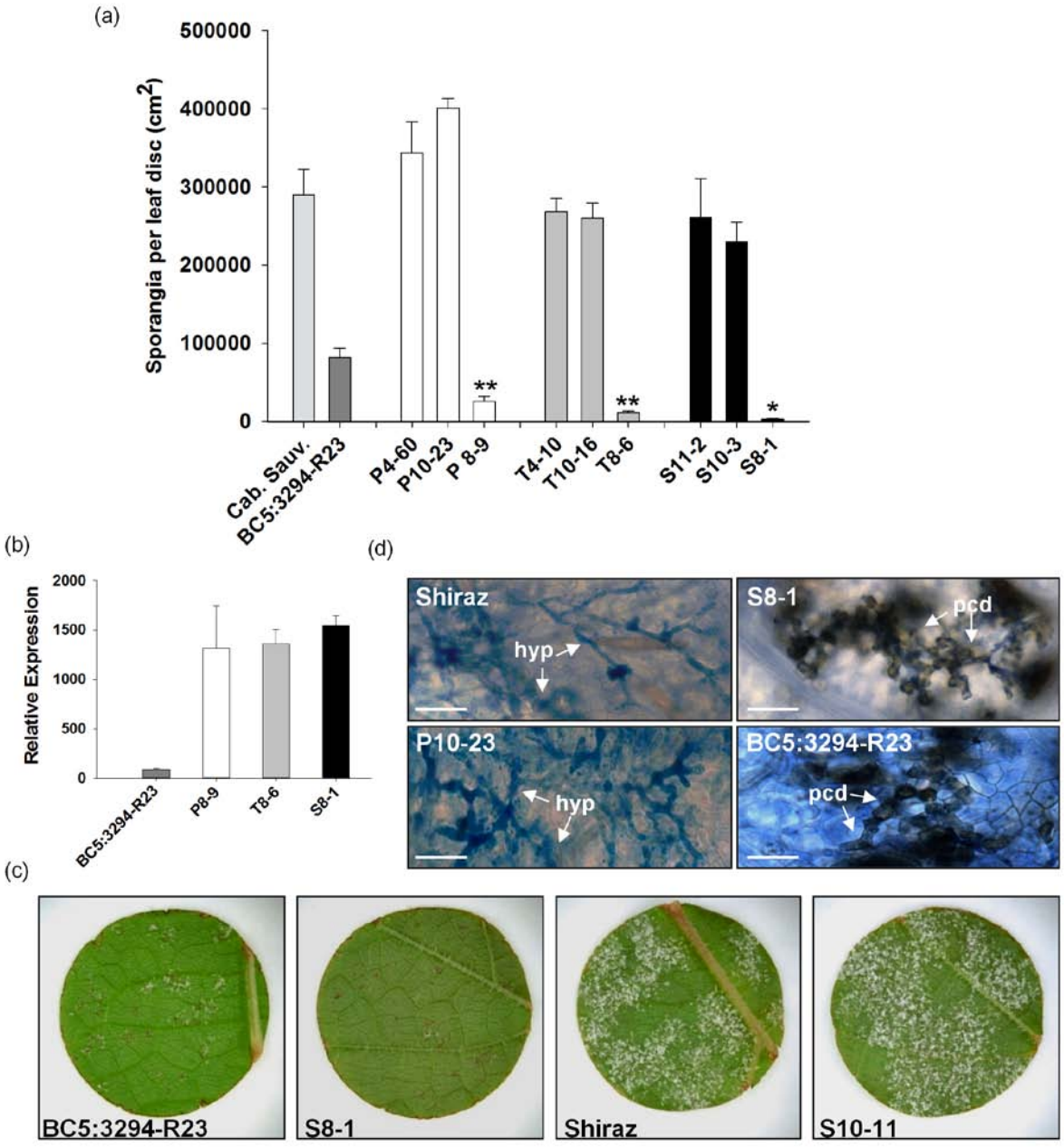


Figure 4. *MrRGA8 (MrRPV1) confers strong resistance to P. viticola in transgenic V. vinifera cultivars. (a) Sporangia per cm^2 area produced 6 dpi of leaf discs sampled from V. vinifera cv. Cabernet Sauvignon, BC5:3294-R23 and individual transgenic lines of V. vinifera cvs. Portan (P), Tempranillo (T) and Shiraz (S) expressing MrRGA4, MrRGA8, MrRGA10 or MrRGA11. Values are means ± SE (n=3). Asterisks indicate a significant difference from Portan, Shiraz or Tempranillo vines transformed with MrRGA4, MrRGA10 or MrRGA11 in the same background (* P< 0.05 and ** P<0.005; Student's t-test). (b) Transcript levels of MrRGA8 normalised to the expression of the housekeeping gene EF1. Each data point is the mean ±SE of triplicate testing of two biological replicates. (c) Downy mildew sporulation on BC5:3294-R23, non-transformed Shiraz and Shiraz transgenic lines expressing MrRGA8 or MrRGA10. (d) Trypan blue staining of downy mildew infected leaf discs. Susceptible lines (Shiraz, P10-23) show extensive hyphal (hyp) development within mesophyll cells whereas most penetrated cells of resistant lines (S8-1, BC5:3294-R23) have undergone PCD. Scale bars = 25µm.*

M*rRUN1 and MrRPV1 generate multiple alternatively spliced transcripts*

A common feature of the transcription of a number of *R*-genes is alternative splicing (Gassmann, 2008). PCR amplification of *MrRGA* transcripts from cDNA of *MrRGA8* or *MrRG10* transgenic lines and the BC5:3294-R23 line indicated the presence of four transcripts (Figure 6). Cloning and sequencing confirmed that both genes produce four different transcripts arising from combinations of different splicing mechanisms (Figure 6a & b) and that each of these four transcripts could potentially produce a different translation product (Figure 6c). Transcript A encodes the full-length protein. Transcript B is generated by the splicing of a cryptic intron from exon 2. Interestingly, the position and size of this cryptic intron varies in transcript B between *MrRGA10* and *MrRGA8*. In both cases, this encodes a protein with a truncation of the NB-ARC domains. Transcript C is generated by splicing between an alternative 5' splice site in exon 2 and the 3' splice site of intron 2. This causes a frame-shift and a premature stop codon resulting in the potential generation of a truncated TIR-NB protein. Finally, transcript D is generated by the complete loss of exon 2 via exon skipping, which encodes a truncated TIR protein.

Cultivated grapevine has a cluster of RGAs at the syntenous region on chromosome 12

The *MrRUN1/MrRPV1* locus in *M. rotundifolia* maps to a region on Chr 12 between the SSR markers VMC4F3-1 and VMC8G9 (Barker *et al.*, 2005). Comparison of the gene organisation and coding sequences between the wild North American species *M. rotundifolia* and the European cultivated grapevine *V. vinifera* reveals that the *MrRGAs* at the *MrRUN1/MrRPV1* locus are most closely related to a cluster of *TIR-NBS-LRR* genes in a syntenic region of *V. vinifera* Chr 12 (Figure 7).

Phylogenetic analysis of the nucleic acid coding regions of the genes at these syntenous locations (Figure 8) suggests that the *V. vinifera TIR-NB-LRR* genes at this locus share a common ancestor with the *MrRGA*s. However, it would appear that following the split of *Vitis* and *Muscadinia*, the selection pressure applied by the presence of the *E. necator* and *P. viticola* pathogens in North America has led to the evolution of the *MrRUN1* and *MrRPV1* resistance genes in the Muscadines which are absent in *V. vinifera.*

NLS

```
MrRUN1: TLGLLG--NFHDNGSAVLEDTLGNRKRRRDDSLPDVVEEPHYKKIGSHPTPISCFELHHQYQSEQNHMW :1331
MrRPV1: LLDLLRNLSLGDNGSVVLEDTLGNRKRRRNDSLPDVVEEPLYKRLGGPHTEISL--------------- :1398
```

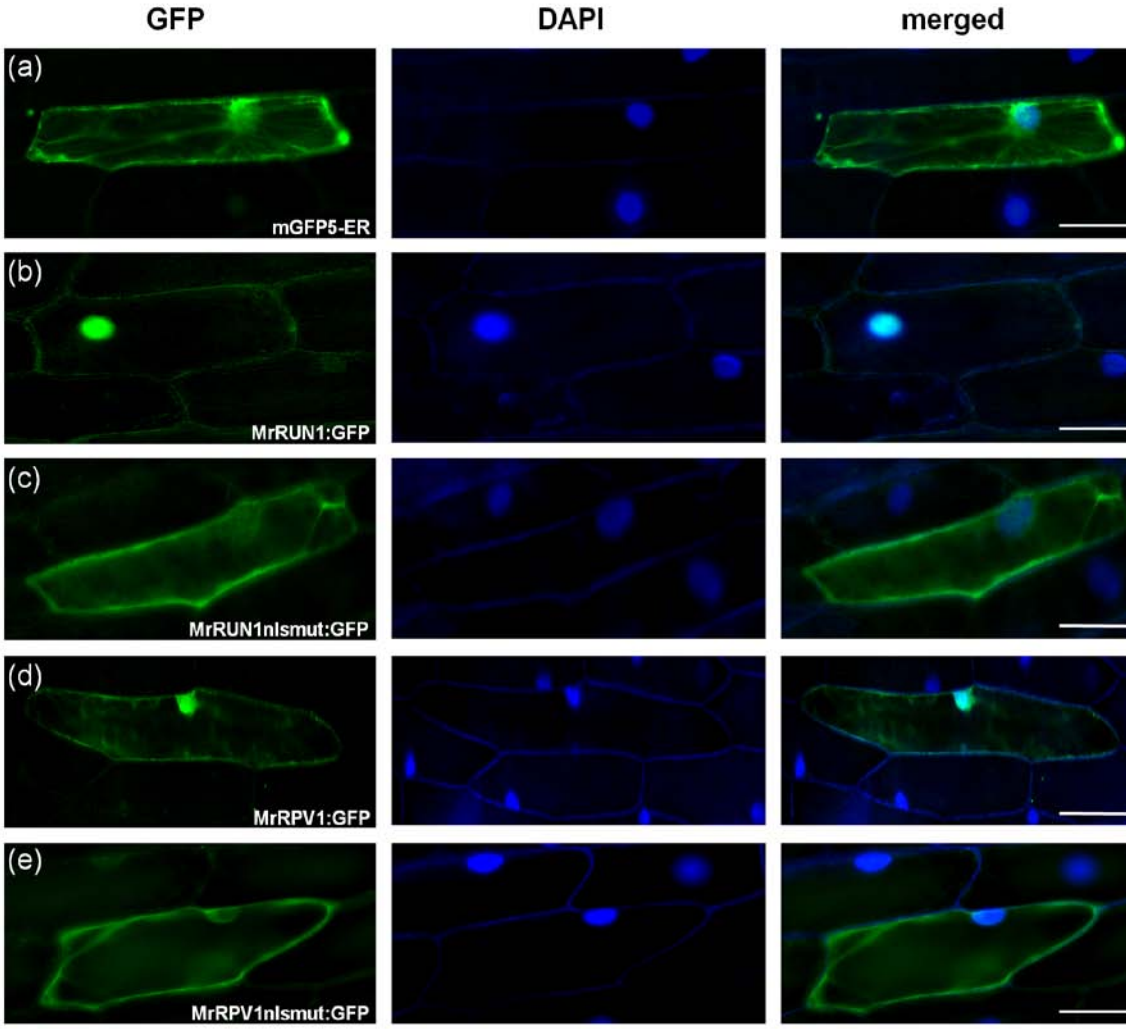


Figure 5. *The sub-cellular localisation of MrRUN1 and MrRPV1 requires a nuclear localisation signal. Top panel: alignment of C-terminal end of MrRUN1 and MrRPV1 proteins with conserved predicted nuclear localisation signal (NLS). Bottom panel: GFP fusion constructs were bombarded into onion epidermal cells and visualized (a-e) after 48 h. The same cells were treated with DAPI stain to visualize nuclei and merged with the GFP images. MrRUN1:GFP (b) and MrRPV1:GFP (d) are targeted to the nucleus in comparison to the endoplasmic reticulum-targeted mGFP-ER fusion protein (a). Mutation of the nuclear localisation signal RKRRR to RTSRR in MrRUN1nlsmut:GFP (c) and MrRUN1nlsmut:GFP (e) prevents nuclear localisation. Scale bars = 100µM*

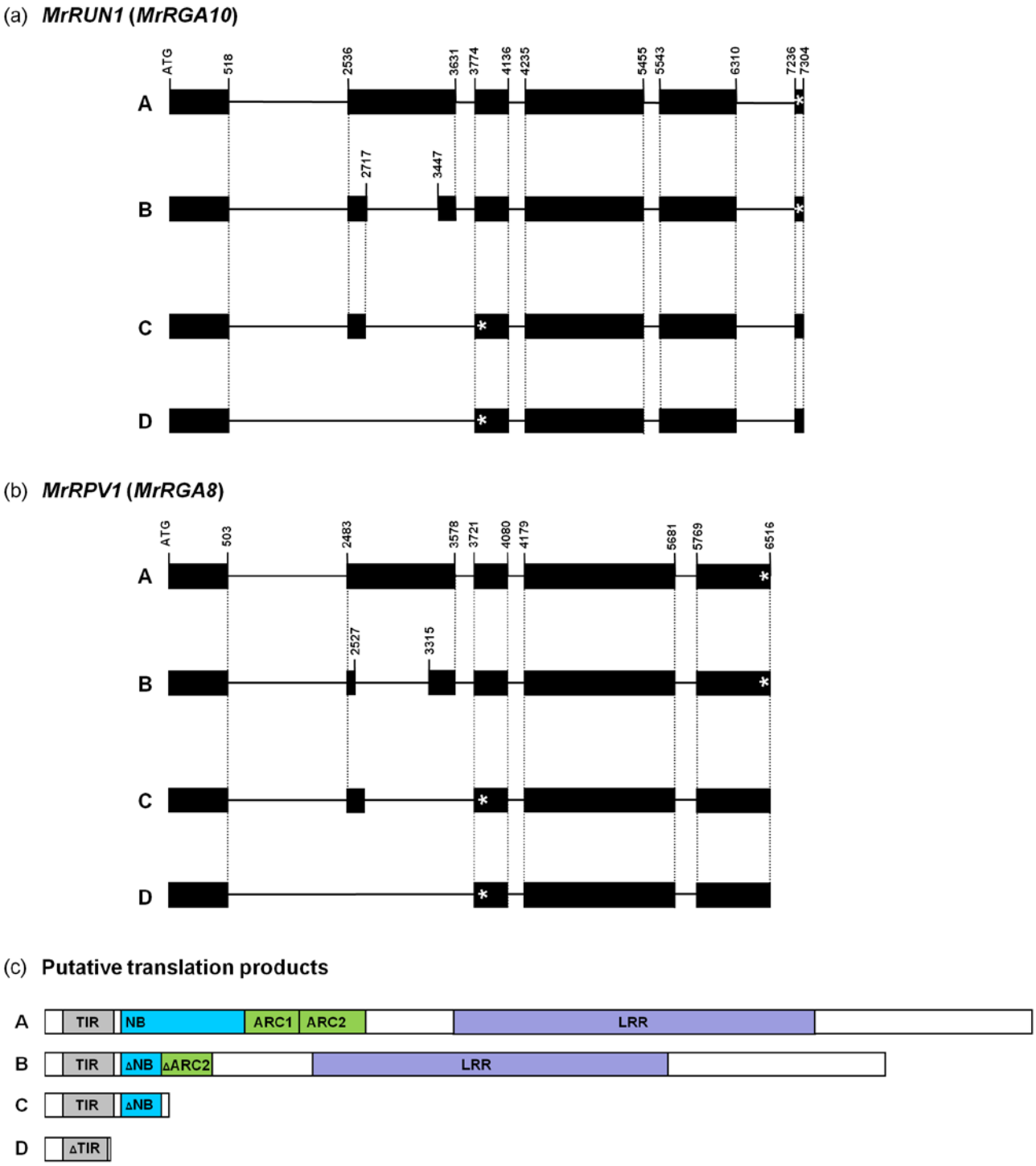


Figure 6. *MrRUN1 and MrRPV1 transcripts undergo alternative splicing. (a, b) MrRUN1 and MrRPV1 undergo alternative splicing via a combination of different mechanisms involving splicing of a cryptic intron from exon 2 and alternative 3' splice sites to produce four transcripts designated A-D. Alternative splice variants of MrRUN1 and MrRPV1 were also confirmed in cDNA isolated from BC5:3294-R23. Stop codons are indicated with an asterisk. Note that alternative splicing in transcripts C and D results in a frame-shift and a premature stop codon. (c) Putative translation products of MrRUN1 and MrRPV1 alternative transcripts: transcript A - full-length protein; B - truncated NB-ARC domain but intact TIR and LRR domains; C - completely lacking the ARC and LRR domains; D - truncated TIR domain only. Δ indicates a protein domain that has been truncated as a result of alternative splicing.*

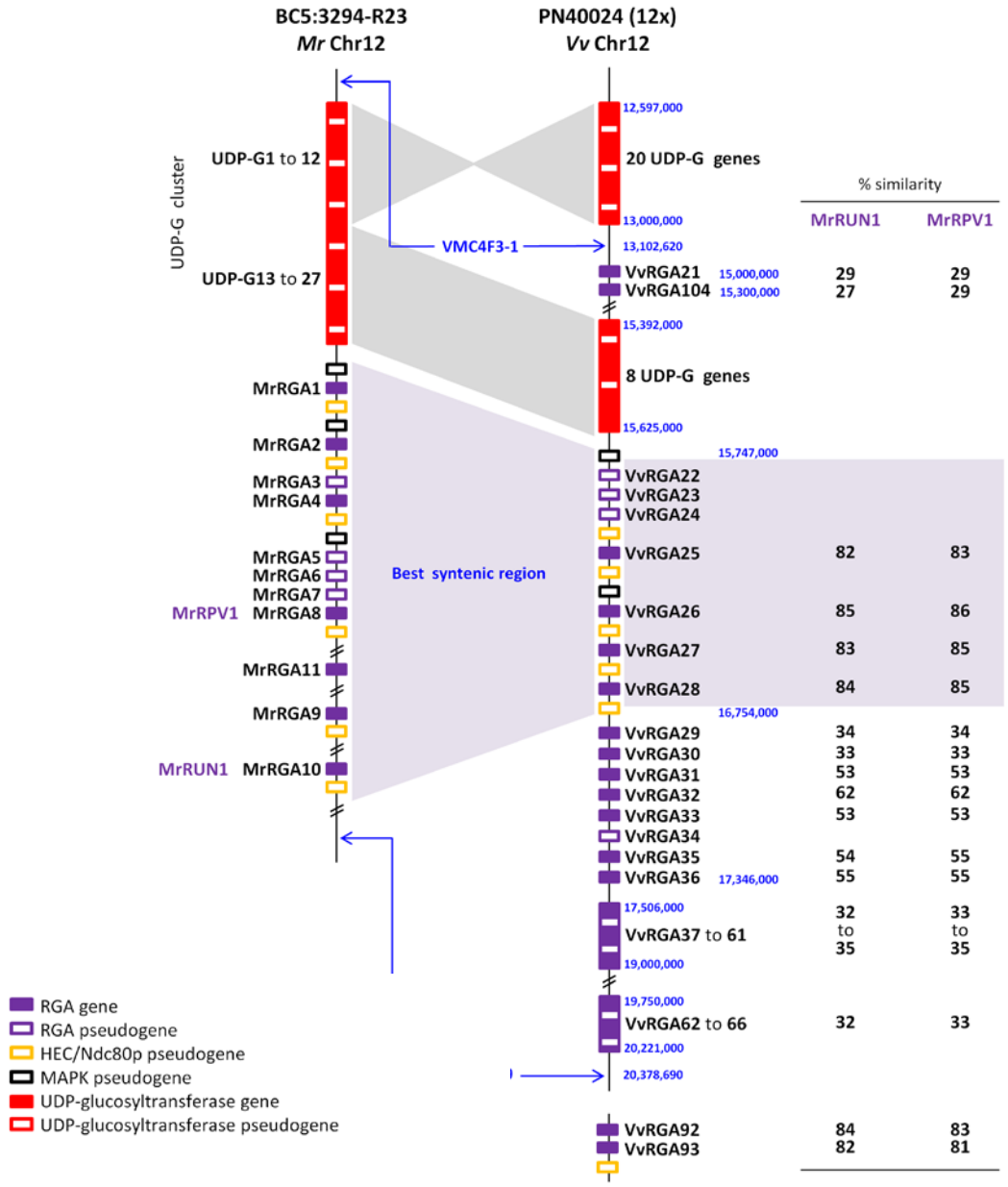


Figure 7. *Similarities between the M. rotundifolia RUN1/RPV1 locus and the homologous region of V. vinifera chromosome 12. Based on the gene organization and the global protein sequence alignments (the levels of amino acid similarity are indicated in the right-hand table), the best syntenic region of the MrRUN1/MrRPV1 locus (highlighted in purple) has been identified between the SSR markers VMC4F3-1 and VMC8G9. Note: pseudogenes are represented by solid and blank colored boxes respectively. Large red and purple rectangles depict clusters (mix of genes and pseudogenes) of genes encoding UDP-glucosyltransferases and RGAs respectively.*

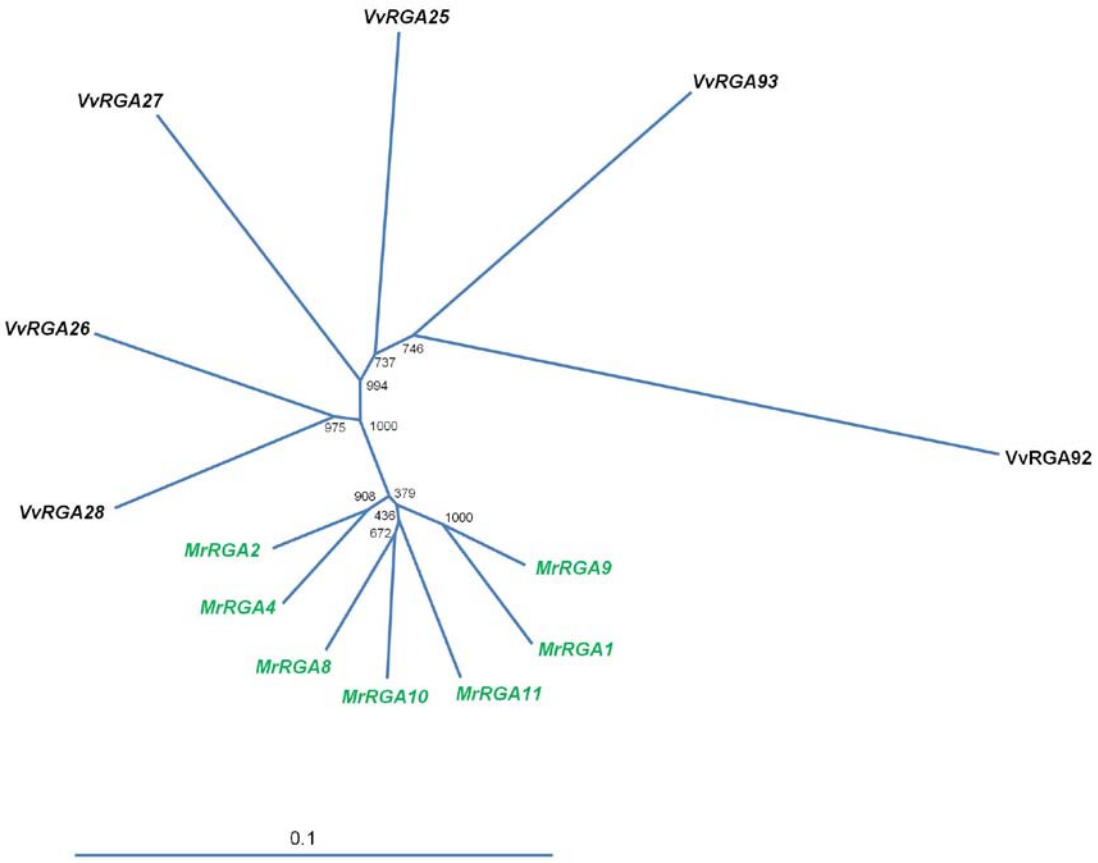


Figure 8. *Phylogenetic relationship between the coding sequences of MrRGAs and VvRGAs*
Nucleic acid sequences encoding MrRGAs from the MrRUN1/MrRPV1 locus of M. rotundifolia were aligned with the most closely related VvRGAs from V. vinifera cv. PN40024 including those from the best syntenic region using CLUSTALW and a phylogenetic tree constructed using the neighbour-joining method. Numbers indicate bootstrap values (from 1000 trials) that support the respective branch. The scale bar represents 0.1 substitutions per nucleotide site.

Assessment of resistance in winegrape breeding lines containing the MrRUN1/MrRPV1 locus

This section of the final report is commercial in confidence and will not be available until after June 2015. If you have any questions please contact the GWRDC

Discussion

MrRUN1 and *MrRPV1* are the first resistance genes to be cloned and functionally characterised from grapevine. We have shown that these resistance genes can be transferred from a wild grapevine species (in this case *M. rotundifolia*) into premium *V. vinifera* cultivars by genetic modification and confer strong resistance against the two major pathogens of cultivated grapevines worldwide.

Despite conferring resistance to two very different pathogens i.e. a fungus and an oomycete, MrRUN1 and MrRPV1 show a high degree of sequence homology (86% amino acid identity) with the major difference being the number of LRR domains (Feechan *et al*., 2013). The LRR domain of rust resistance proteins has been shown to be a major determinant of resistance specificity (Ellis *et al.*, 2007) and may also explain the different pathogen specificities of MrRUN1 and MrRPV1. The observation that paralogous members of the same NB-LRR gene family, within the same genotype, provide resistance to two unrelated pathogens is unusual, but not unique. A similar example is the *Rx* / *Gpa2* R-gene cluster in potato where two homologues with 88% amino acid identity provide resistance to a virus and a nematode (van der Vossen *et al.*, 2000). Similarly, it has been recently been reported that resistance to powdery mildew and root-knot nematodes in tomato is conferred by homologues within the same *Mi-1* gene cluster (Seifi *et al.*, 2011). We also cannot rule out the possibility that one of the other five full-length *MrRGA*s at the *MrRUN1*/*MrRPV1* locus may act either individually or through co-operative interaction (Narusaka *et al.*, 2009) with *MrRUN1* or *MrRPV1*, to confer resistance to other grapevine pathogens.

Interestingly, although levels of PCD induction observed in transgenic *MrRUN1* vines were sufficient to confer resistance to powdery mildew, they were generally lower than that observed in the BC5:3294-R23 genotype from which the *MrRUN1* gene was isolated (Figure 2a, S2a). This could not be ascribed to differences in the level of *MrRUN1* transcription in the transgenic lines relative to BC5:3294-R23 (Figure 2b). Most likely, this probably reflects the minor contributions of other *RGAs* at the *MrRUN1/MrRPV1* locus, such as *MrRGA4,* to the total level of powdery mildew resistance observed in the BC5:3294-R23 genotype (Figure S2). Conversely, the *MrRPV1* transgenic vines displayed significantly higher levels of downy mildew resistance than BC5:3294-R23 (Figure 3a) which may be due to the much higher levels of *MrRPV1* transcription in transgenic vines relative to the parental line (Figure 3b). We also cannot rule out the possibility that that the genetic background of the *V. vinifera* cultivar may have a significant influence on the expression of resistance mediated by the Muscadine genes.

It has previously been shown that disease-resistance mediated by certain TIR-NB-LRR resistance genes requires the combined presence of both full-length and truncated open reading frames. Both the *N* gene in tobacco (Dinesh-Kumar and Baker, 2000) and the *RPS4* gene in Arabidopsis (Zhang and Gassmann, 2003) failed to exhibit complete resistance in the absence of the alternatively spliced transcripts. The alternative transcripts produced from *MrRUN1* and *MrRPV1* encode the full-length protein and three putative truncated products (Figure S8). We are currently in the process of generating grapevine transgenics expressing full-length *MrRUN1* and *MrRPV1* cDNA constructs, to investigate the role of these putative truncated proteins in disease resistance. It is conceivable that the different *MrRUN1* and *MrRPV1* transcript products may have different roles in defence or PCD signalling dependent on their cellular localisation. For example, it has recently been proposed that while the

nuclear pool of effector-activated MLA10 powdery mildew resistance protein triggers disease resistance signalling, the cytoplasmic pool of MLA10 is involved in cell death signalling (Bai *et al.*, 2012). Thus, the full-length MrRUN1 and MrRPV1 proteins that are targeted to the nucleus (Figure 4) may have a role in disease resistance signalling, while the putative truncated TIR-NB protein generated from transcript C (Figure S8), which encodes a full plant TIR domain (Bernoux *et al.*, 2011) but lacks the NLS, may be important for cytoplasmic cell death signalling. Future experiments involving transgenic grapevines expressing full-length MrRUN1 and MrRPV1 proteins lacking an NLS and fused to a nuclear export signal (NES) at the C-terminus to completely exclude these R proteins from the nucleus, as was previously achieved with MLA10 (Shen *et al.*, 2007), will help resolve the question of the biological significance of the nuclear localisation of these proteins.

In addition to the demonstration that *MrRUN1* and *MrRPV1* can be successfully transferred into premium winegrape varieties by Agrobacterium-mediated transformation we have also demonstrated in GWRDC project CSP 0904 that the *MrRUN1*/*MrRPV1* locus can be introgressed into premium winegrape varieties by classical hybridization. Interestingly, some minor differences in resistance levels between progeny from different parents were detected with the *in vitro* assays. However, it is important to remember that the level of mildew inoculum applied in the *in vitro* assays is much higher than a plant would normally experience in the field and the conditions under which the leaf tissue is incubated are much more favorable for pathogen development than would be normally present in the field. Thus, the small reductions in resistance observed in the *in vitro* assays, with some genotypes, are not expected to be translated into susceptibility in the field under normal conditions and, to date, this has been confirmed by the excellent performance of these vines in unsprayed blocks at Nuriootpa.

6. Evaluation of resistance of *MrRUN1* & *MrRPV1* transgenics against powdery and downy mildew isolates from Europe and North America

Introduction

It was recently reported that *E. necator* isolates in North America (with the exception of California where only group B isolates are found) are more genotypically diverse than those in Europe and Australia (Brewer and Milgroom, 2010). This is consistent with *E. necator* being endemic to this region. In barley, specific *Mla* alleles provide race-specific resistance (Zhou *et al.*, 2001). Therefore, in addition to examining the isolate-specificity of *MrRUN1*, we also wanted to determine if any of the other *MrRGA* genes confer resistance to specific isolates in North America. To determine the isolate-specificity of the resistance conferred by *MrRUN1*, transgenic lines were challenged with *E. necator* isolates from different genetic groups and geographical locations. Much less is known about the relatedness or otherwise of grapevine downy mildew isolates. However transgenic lines were challenged with *E. necator* isolates from Europe and North America.

Materials and methods

Erysiphe necator culture and inoculations were performed as previously described (Donald *et al.*, 2002) using isolates Chlo2b (group B, collected at Pauillac, France), LNYM (haplotype 28, collected from Lockport, NY), Musc4 (group M, collected from Georgia) and NY137 (collected from Geneva, NY). *Plasmopora viticola* inoculations in France were performed at INRA-Colmar as described previously (Blasi *et al*., 2011) with either the SC or SL isolate (Peressotti *et al.*, 2010). In North America, inoculations were performed at the Department of Horticulture, Cornell University, Geneva, NY with a *P. viticola* isolate (NYR) collected from an experimental vineyard in Geneva.

In vitro cuttings of the parental line BC5:3294-R23, untransformed *V. vinifera* cultivars Maccabeu, Portan, Tempranillo (T) and Shiraz (S) and at least two independent *MrRGA* transgenic lines for each *MrRGA* construct were sent to Dr Bruce Reisch, Horticulture Dept, New York Agricultural Station, Cornell University, Geneva, NY and established in growth chambers for inoculation and analysis by Dr Angela Feechan. RGA transgenics to be tested against European isolates were transferred from the transformation laboratory of Dr Laurent Torregrosa, ENSA-M/INRA, Montpellier, France to Dr Pere Mestre, INRA Colmar, France for inoculation and analysis. Powdery mildew and downy mildew assays were conducted as described previously (Page 17).

Results

Screening of MrRGA transgenics for resistance against European and North American and powdery mildew isolates

Figures 11 and 12 show the results of screening *MrRGA* transgenic vines with an Australian *E. necator* group A isolate. *MrRGA10* transgenics also showed complete resistance to a group B powdery mildew isolate obtained from a French vineyard (Blanc *et al.*, 2012) (Figure 11). Selected *MrRGA* transgenic lines were also challenged with three genetically diverse North American *E. necator* isolates which fall into genetic groups distinct from the A and B types already tested: LNYM and NY137 collected from New York and Musc4 collected from Georgia (Brewer and Milgroom, 2010)

As observed with *E. necator* isolates from Australia and France, only transgenic lines expressing *MrRUN1* (*MrRGA10*) were found to confer resistance to the powdery mildew isolates LNYM and NY137 (Figure 12a & b). However, what was particularly significant was the finding that neither transgenic lines expressing *MrRUN1* (*MrRGA10*) alone, nor the BC5:3294-R23 line containing the entire *MrRUN1/MrRPV1* locus, induced a resistance response to inoculation with the Musc4 isolate from Georgia (Figure 12c) indicating that this isolate was capable of breaking the resistance conferred by *MrRUN1*.

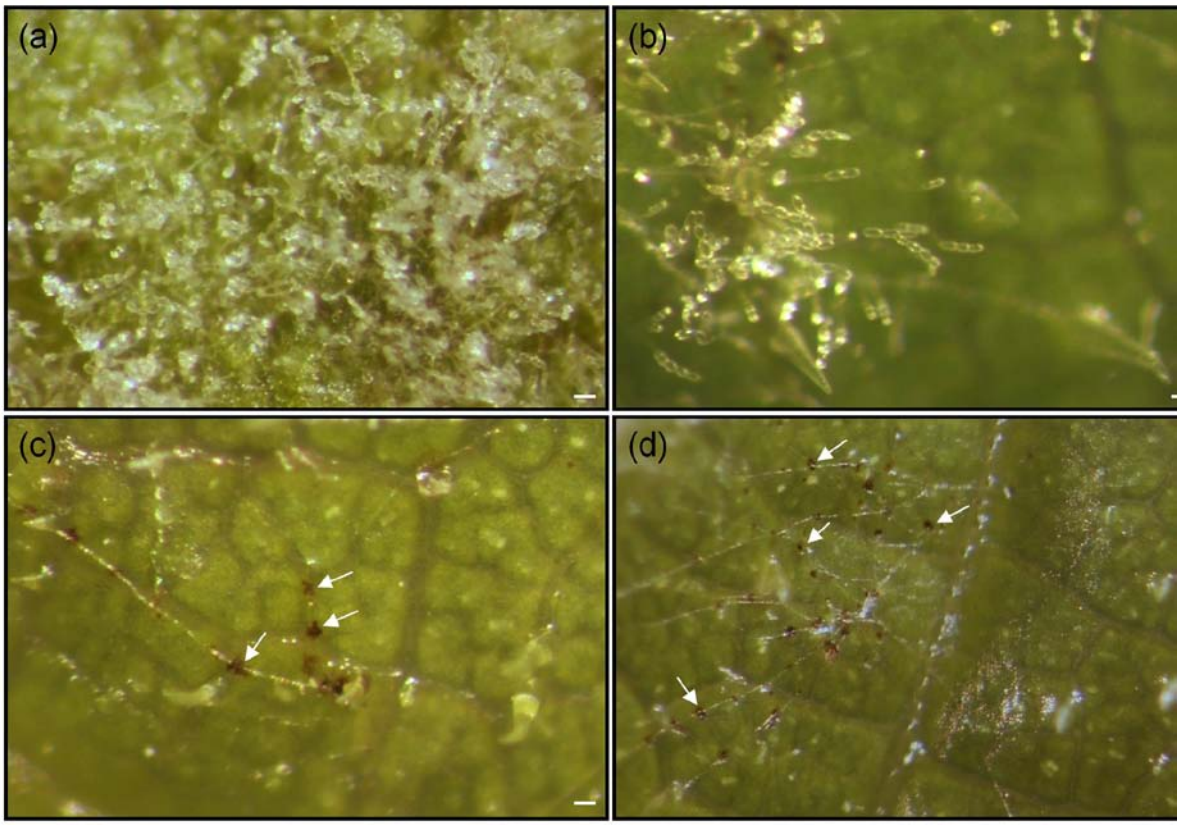


Figure 11. *Transgenic V. vinifera cultivars expressing MrRUN1 are resistant to a French powdery mildew isolate. Detached leaves collected from glasshouse-grown vines were inoculated with the French E. necator isolate Chlo2b. Leaves were sampled 9 dpi. Resistance was only observed in transgenic vines expressing MrRUN1 (MrRGA10). Data is shown for (a) untransformed V. vinifera cv. Muscat Ottonel (b) MrRGA8 transgenic line of V. vinifera cv. Tempranillo, T8-6 (c) MrRGA10 transgenic line of Tempranillo, T10-44 (d) MrRGA10 transgenic line of V. vinifera cv. Carignan, C10-5. Images are representative of infection of two leaves from two independent vines for each line shown. White arrows indicate epidermal cells which have undergone pcd following penetration. Scale bars=50µM.*

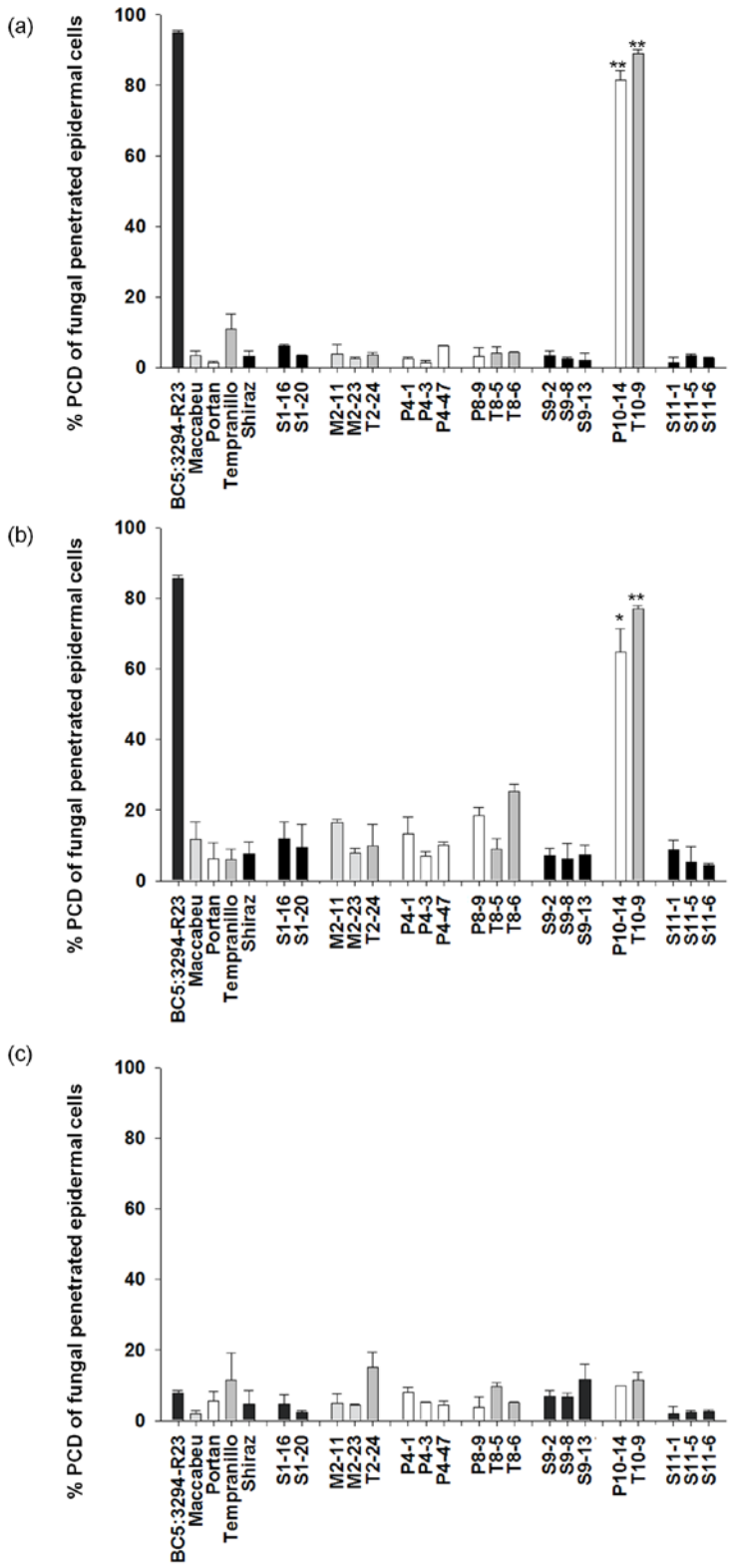


Figure 12. *Screening of transgenic V. vinifera cultivars expressing individual MrRGAs for resistance to North American powdery mildew isolates. Detached leaves collected from glasshouse-grown vines were inoculated separately with three different North American E. necator isolates: LNYM (a), NY137 (b) or Musc4 (c). Leaves were sampled 48 hpi and scored for the induction of PCD in powdery mildew-penetrated epidermal cells using trypan blue staining. Data is shown for the powdery mildew resistant BC5:3294-R23 line and both untransformed and MrRGA transgenic lines of V. vinifera cultivars Maccabeu (M), Portan (P), Tempranillo (T) and Shiraz (S). A minimum of two independent transgenic lines were tested for each MrRGA construct. Each data point is the mean ± SE (n=2). * indicates that P<0.01, ** indicates that P<0.005.*

recently identified in the Czech Republic, that has been shown to break resistance conferred by an alternative resistance locus, *RPV3,* from the complex North American Vitis hybrid 'Bianca' (Peressotti *et al.*, 2010) (Figure 14c, d). This demonstrates that *MrRPV1* confers strong resistance to a range of different *P. viticola* isolates present in the major winegrape-growing regions of France, North America and Australia.

Discussion

Transgenic winegrape cultivars expressing *MrRUN1* were shown to be resistant to genetically diverse isolates collected from winegrowing regions in Europe, Australia and North America, indicating that MrRUN1 recognises a fungal effector molecule conserved within these geographically distinct isolates. In contrast, the barley *Mla* powdery mildew locus encodes 28 characterized resistance specificities to different isolates of barley powdery mildew (*Blumeria graminis f. sp. hordei*) (Zhou *et al.*, 2001). Similarly, *MrRPV1* was found to confer resistance to four different *P. viticola* isolates found in Europe, North America and Australia including an isolate from the Czech Republic reported to break the alternative *P. viticola* resistance source *RPV3* (Peressotti *et al.*, 2010). This contrasts with the downy mildew resistance locus *Dm* in lettuce where a cluster of genes have evolved to confer race-specific resistance (Meyers *et al.*, 1998). The relatively broad isolate-specificity of the *MrRUN1*/*MrRPV1* locus from *M. rotundifolia*, in contrast to similar resistance loci in other annual crops, most probably reflects the fact that most winegrape regions of the world are planted with highly susceptible *V. vinifera* cultivars where there is minimal selective pressure on the pathogen to evolve new race specificities.

However, *MrRUN1* vines were not able to confer resistance to an isolate (Musc4) collected from the south-eastern region of North America from where Muscadine vines originate (Figure 12c). This indicates that the effector recognised by MrRUN1 has either been lost or mutated in the Musc4 isolate in response to a long period of co-evolution with powdery mildew resistant Muscadine vines. Musc4 belongs to a genetically distinct group of isolates (group M) found only in the South-eastern region of North America (Brewer and Milgroom, 2010), and as such, would not represent an immediate threat to the durability of resistance conferred by *MrRUN1* in the major winegrape-growing regions of Europe, North America and Australia. This observation does, however, highlight the need to identify new sources of genetic resistance to *E. necator* and *P. viticola*, to overcome potential problems associated with the deployment of race-specific single gene resistance and to maximise the durability of disease-resistance vines in the vineyard.

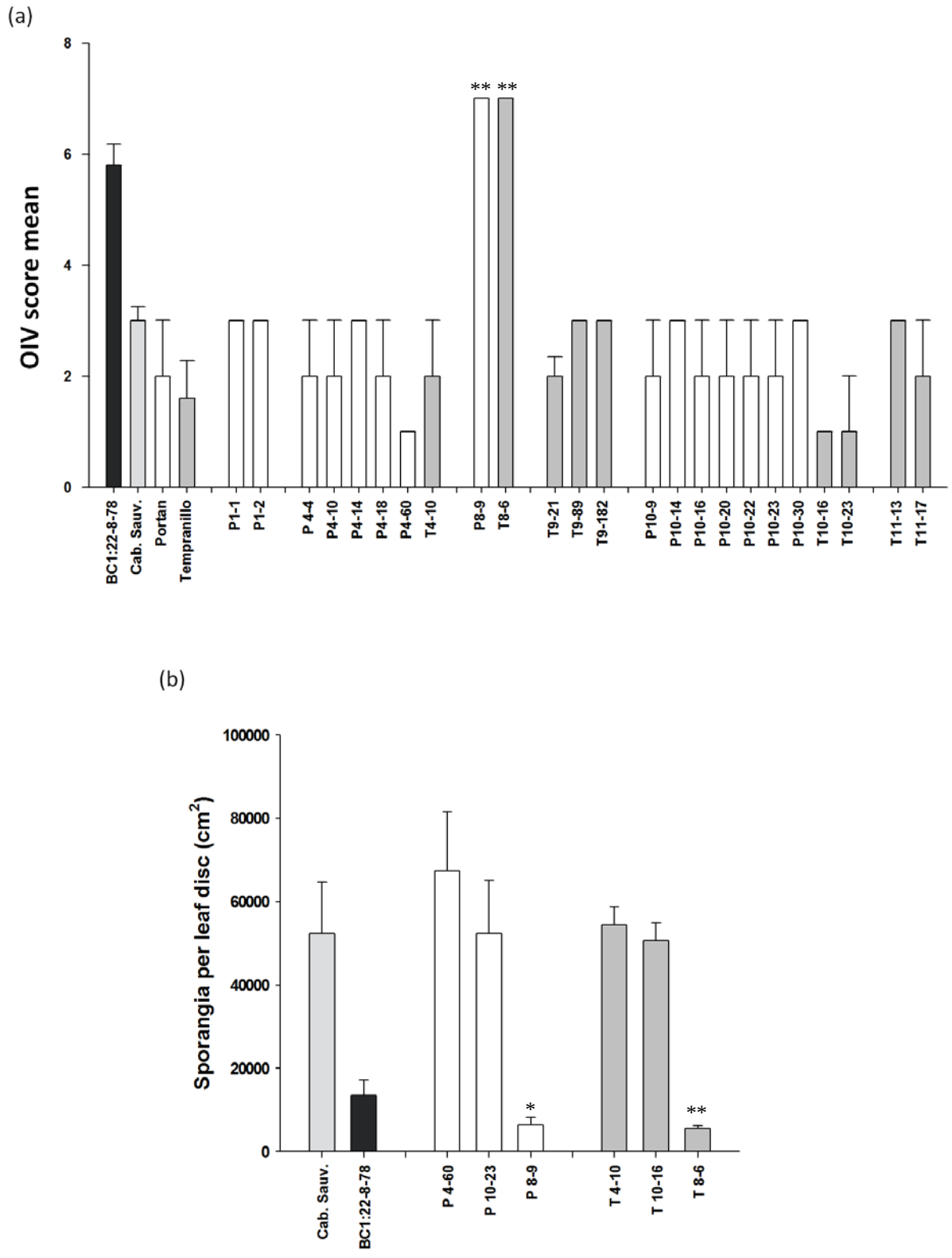


Figure 13. *Screening of transgenic V. vinifera cultivars expressing individual MrRGAs for resistance to a French downy mildew isolate. Leaf disc OIV scores of MrRGA transgenic lines of the V. vinifera cultivars Portan (P), and Tempranillo (T), untransformed Cabernet Sauvignon and downy mildew-resistant lines BC1:22-8-768 inoculated with P. viticola sporangia of the French SC isolate. Downy mildew infection was scored 6 dpi using either the OIV disease index scale (a) or by counting sporangia production (b). OIV scoring as described in Figure 3. Each data point is the mean ± SE (n=2). * indicates P<0.05, ** indicates that P<0.005.*

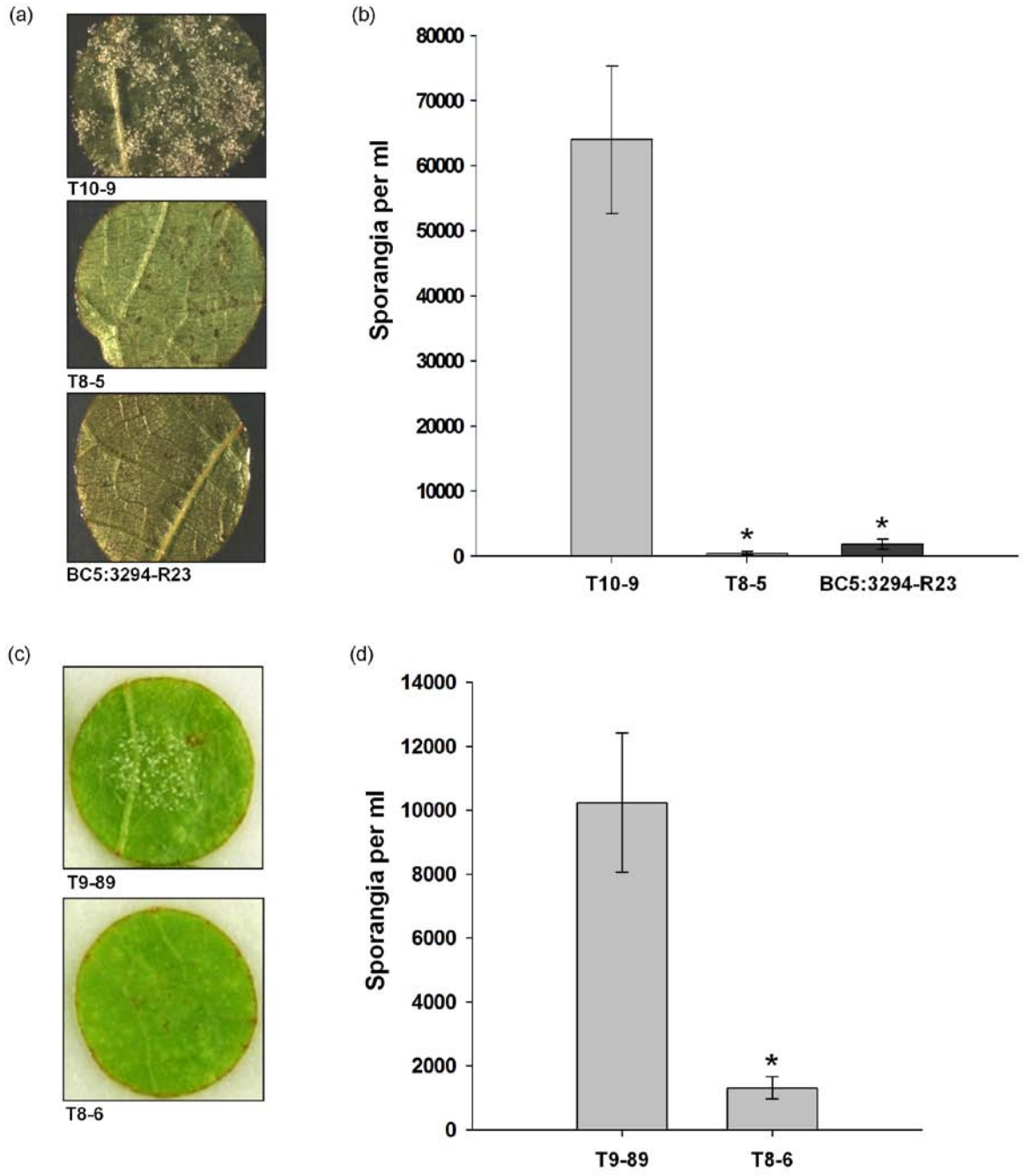


Figure 14. *Transgenic V. vinifera cultivars expressing MrRPV1 (MrRGA8) confer resistance to a North American downy mildew isolate and an RPV3-resistance breaking downy mildew isolate from the Czech Republic. Individual transgenic lines of V. vinifera cv. Tempranillo (T) expressing either MrRGA8 (T8-6, T8-5), MrRGA9 (T9-89), MrRGA10 (T10-9) or the downy mildew resistant line BC5:3294-R23 were inoculated with either a North American isolate NYR obtained from New York (a, b) or the RPV3-resistance breaking isolate SL from the Czech Republic (c, d) and the discs visualised 6 dpi by light microscopy (a, c). Note the presence of necrotic lesions on inoculated discs expressing MrRGA8 and on the resistant line BC5:3294-R23. Sporangia production is shown in (b, d). Values are means ± SE (n=2). Asterisks indicate a significant difference from control (* P< 0.05; Student's t-test).*

7. The search for new resistance genes to grapevine powdery mildew and downy mildew and the development of new transgenic strategies to enable pyramiding with *MrRUN1* & *MrRPV1*

Introduction

Durable resistance can be considered as resistance that remains effective when used in a large growing area, over a long period of time, under environments favourable to disease development (Leach et al. 2001). Perennial crops, like grapevines, provide particular challenges when considering the issue of durability. Most grapegrowers would have an expectation that individual vines would remain in the vineyard for at least 20 years and do not have the capacity to rapidly introduce new cultivars with different *R* genes, as is the case for annual crops such as cereals, should existing *R* genes fail.

To date, there are few examples of single dominant *R* genes that have proven to be durable in the field. Host plant resistance involving these *R* genes can be lost through modification or elimination of pathogen effector(s) that trigger the resistance response. Even the barley *Rpg1* gene which had provided effective control for almost 50 years in North America against stem rust caused by *Puccinia graminis* f. sp. *tritici* was finally compromised by the evolution of a new virulent pathotype (Steffenson, 1992). One approach which may improve the durability of resistance in the field, is to stack or pyramid multiple *R* genes within a single cultivar such that, for a given pathogen, reproduction will be restricted even if individuals within the pathogen population are present that have lost, or modified, the interacting effector molecule for one of the *R* genes. A number of groups have already commenced breeding programs to combine *Run1* resistance with these other powdery mildew resistance loci using marker assisted evaluation to follow the inheritance of both resistance loci (Eibach *et al*,. 2007)

The aim of this component of the project was two-fold. Firstly, to investigate other sources of genetic resistance to powdery and downy mildew that could be used to pyramid with *MrRUN1* and *MrRPV1* within the same genotype to increase the durability of resistance to these pathogens in the vineyard. Secondly, to investigate the feasibility of introducing two such *R* genes simultaneously into the same transgenic line.

Materials and Methods

Plant and fungal material

Muscadine germplasm was obtained from the CSIRO Plant Industry germplasm collection, Merbein Vic, the USDA grapevine germplasm repository in Geneva, New York, USA and Professor Andy Walker, UC-Davis, California, USA.

Vitis candicans canes were sourced from the Coombe vineyard, Waite Campus, University of Adelaide. *Vitis candicans* produces female flowers and these were pollinated with pollen from *V. vinifera* cv. Cabernet Sauvignon. Approximately 200 F1 seeds were collected and these were sterilised and cold-treated for 3 weeks. Seeds were germinated under sterile

conditions in petri dishes on moistened sterile filter paper and then transferred to the glasshouse.

A cross between *Vitis cinerea* (MF2-50) and *V. vinifera* cv. Riesling was previously established at the CSIRO Plant Industry research vineyard at Koorlong, Victoria as part of the rootstock breeding program.

Vitis romanetii and *V. piasezkii canes* were imported from the USDA-ARS National Clonal Germplasm Repository, Davis California. Seed of BC2 population 12-3501 carrying the *Ren4* powdery mildew resistance locus derived from *Vitis romanetii* was obtained from Dr David Ramming, USDA/ARS/CDPG, Parlier, California.

Downy mildew resistance screening

Freshly cut leaf discs were placed onto moist filter paper in Petri dishes abaxial side up and inoculated with a 20µl droplet of *P. viticola* suspension (5×10^4 sporangia/ml) overnight. After the droplet was removed, the leaf discs were maintained in a temperature-controlled incubator at 22-23^{o}C with a 16 h light / 8 h dark light cycle. Two replicates were performed for each individual of the mapping population and 10 discs for each replicate. A quantitative evaluation of sporulation was obtained 6 days after inoculation by measuring the number of sporangia per disc for each plant with a Fuchs-Rosenthal Haemocytometer.

Powdery mildew resistance screening

Programmed cell death-mediated powdery mildew resistance in penetrated epidermal cells was carried out as described previously (Page 17). Resistance to powdery mildew penetration was assayed as follows: glossy, mature leaves were surface-sterilising and inoculated with *E. necator* spores. After 48 h incubation, infected leaves were stained in a trypan blue solution, destained in chloral hydrate and then mounted onto slides for powdery mildew visualization. Penetration resistance was assessed by counting 100 germinated spores and categorising them based on whether the spore had produced an appresorium but failed to penetrate or whether the fungus had produced an appresorium and successfully penetrated the epidermal cell as indicated by the presence of a visible haustorium or the production of secondary hyphae.

Grapevine transformation with dual resistance genes

Embryogenic *V. vinifera* cv. Shiraz callus was initiated and maintained using media and methods as described by Iocco *et al*. (2001).

Strategy A: Dual R genes in a single binary transformation construct

(a) pCLB2301-RGA8 cDNA

The full-length RGA8 coding region was amplified in two fragments from cDNA made from an RGA8 transgenic grapevine. The first product was amplified by primers CB319 and CB568 generating a 3.9 kb fragment which was sub-cloned into pCR-Blunt. A second product (~1kb) was amplified with primers CB563 and RGA8 3'-*Sbf* I/*Kpn* I-R1 and was also cloned into pCR-Blunt. A *Bsm* I/*Kpn* I digest released the smaller fragment which was cloned into the 3' end of the 3.9kb RGA8 cDNA pCR-Blunt. This RGA8 cDNA fragment was sub-cloned as a *Stu* I/*Sbf* I fragment into the binary vector pCLB2301-RGA8delR which had also been digested with *Stu*

I/*Sbf* I to release the RGA8 genomic sequence to generate the binary vector pCLB2301-RGA8 cDNA.

(b) pCLB2301NK-RGA10 cDNA

Primers RGA10 B/C-F and RGA10 3' *Sma* I/*Sbf* I-R1 were used to amplify a ~3.6 kb product from cDNA made from an RGA10 transgenic grapevine which was cloned into pCR-Blunt. Clone pCR-Blunt RGA10 cDNA was digested with *Stu* I/*Sma* I, releasing a 3.9kb fragment which was then cloned into the binary vector pCLB2301NK-RGA10F which had also been digested with *Stu* I/*Sma* I to release the RGA10 genomic sequence creating pCLB2301NK-RGA10 cDNA

(c) pCLB2301-RGA8 cDNA + RGA10 cDNA

To combine both resistance gene cDNAs in the same binary vector, *pCLB2301NK-RGA10cDNA* was digested with *Sbf* I which released the ~5.5kb cassette of RGA10 cDNA. This fragment was then cloned into the *Sbf* I site of pCLB2301-RGA8 cDNA to generate pCLB2301-RGA8 cDNA + RGA10 cDNA.
To confirm the presence of the RGA8 cDNA and RGA10 cDNA constructs in both the single and double cDNA transgenics, we isolated total RNA for cDNA synthesis (as described on page 17) and confirmed transcription of the transgenes using primers CB20 and CB632.

Strategy B: Simultaneous transformation with different constructs containing R genes using mixed Agrobacterium culture

An 11.5 kb *Xho* I genomic clone of RGA10 was cloned into *Sal* I-digested pCAMBIA2300 to produce pCAMBIA 2300-RGA10. Transformation with dual agrobacterium cultures was essentially the same as for single agrobacterium cultures except that the two Agrobacterium cultures were mixed in equal proportions immediately prior to the co-cultivation step. Callus was selected on kanamycin at a concentration of 150μg/ml until 8 weeks post-infection after which it was reduced to 100μg/ml. Green fluorescent protein (GFP) expression was visualised using a Leica MZ12 microscope with a GFP PLUS Fluorescence attachment. Embryos that germinated on kanamycin and also expressed green fluorescent protein (GFP) were selected for further propagation and finally transferred to the glasshouse for phenotyping.

To confirm the presence of both the RGA8 and RGA10 genomic constructs in transgenic grapevines transformed by the dual Agrobacterium transformation method a small-scale genomic DNA preparation was made from *in-vitro* plants. The quality of the purified DNA was first validated by PCR using elongation factor-1 primers (EF1F/R) before specific primers were used to check for RGA8 (CB600 & CB632) and RGA10 (AJ102 & AJ103).

Results and Discussion

Screening of Muscadine genotypes for new powdery and downy mildew resistance loci

A number of genotypes in the Merbein germplasm collection which were known to have Muscadinia parentage were screened for powdery and downy mildew resistance to identify possible new sources of resistance that could be used to pyramid with *MrRUN1/MrRPV1* in new grapevine cultivars. Seven individual genotypes were found to have strong resistance to both powdery and downy mildew. DNA genotyping was undertaken with the single

nucleotide polymorphism (SNP) markers *VVMT14* and *VVMT16* (developed as part of GWRDC project CSP 0904 to screen for progeny containing the *MrRUN1/MrRPV1* locus) revealed that five of the seven genotypes were, in fact, pure Muscadine species and two were Muscadinia x Vitis hybrids. It is well known that obtaining fertile hybrids between Muscadinia and Vitis are very difficult because of the different number of chromosomes of each species (Vitis 2n = 38, Muscadinia 2n = 40). Therefore, no further work was undertaken with these five pure Muscadine genotypes.

The remaining two Muscadinia hybrids, DRX55 and Ms27-31, were further analysed for the presence of the *MrRUN1/MrRPV1* locus to determine if the observed mildew resistance was likely to be due to the presence of this locus. Results indicated that the DRX55 genotype appears to contain the *MrRUN1/MrRPV1* locus (Table 2) and was therefore not pursued any further. In contrast, Ms27-31, appeared to lack many of the RGA genes found at the *MrRUN1/MrRPV1* locus suggesting that the observed resistance to powdery and downy mildew may be derived from a different resistance locus other than *MrRUN1/MrRPV1*.

Table 2. *Screening of Muscadine genotypes with PCR markers specific to the RGA genes present in the MrRUN1/MrRPV1 locus. Primer sequences are listed in Appendix 5.*

Genotype	RGA1	RGA2	RGA4	RGA8	RGA9	RGA10	RGA11
	RGA1-F1 RGA1-R1	RGA2-F2 RGA2-R2	RGA4-F1 RGA4-R1	RGA8-F1 RGA8-R1	RGA9-F1 RGA9-R1	RGA10-F1 RGA10-R1	RGA11-F1 RGA11-R1
DRX55	✓	✓	✓	✓	✓	✓	✓
Ms27-31	×	×	×	×	×	✓	✓

In order to identify the location of these putative new powdery and downy mildew resistance loci, numerous attempts were made to hybridize Ms27-31 with the *V. vinifera* picovine. Unfortunately none of these attempts were successful, possibly due to the low fertility of Ms27-31 pollen. Low pollen fertility of F1 hybrids is known to be one of the major difficulties in introgressing economically useful characters in a range of crops.

In another set of experiments carried out by Dr Feechan while at New York Agricultural Experimental Station in Geneva, NY, a collection of Muscadine genotypes were challenged with three different North American isolates. Based on other genetic evidence (data not shown), it is thought that these different Muscadine genotypes may carry up to three different *MrRUN1* alleles. The results show that these three different *MrRUN* alleles induce strong PCD in cells penetrated by isolates LYNM and Run137 (Figure 15). However, the resistance conferred by all three alleles is broken by the Musc4 isolate (Figure 15).

Thus, in light of the difficulty in using the Ms27-31 material for generating segregating populations and the finding that resistance conferred by *MrRUN* alleles from different *Muscadinia rotundifolia* cultivars is broken by the Musc4 isolate, a decision was made to search for new sources of resistance outside the Muscadine family.

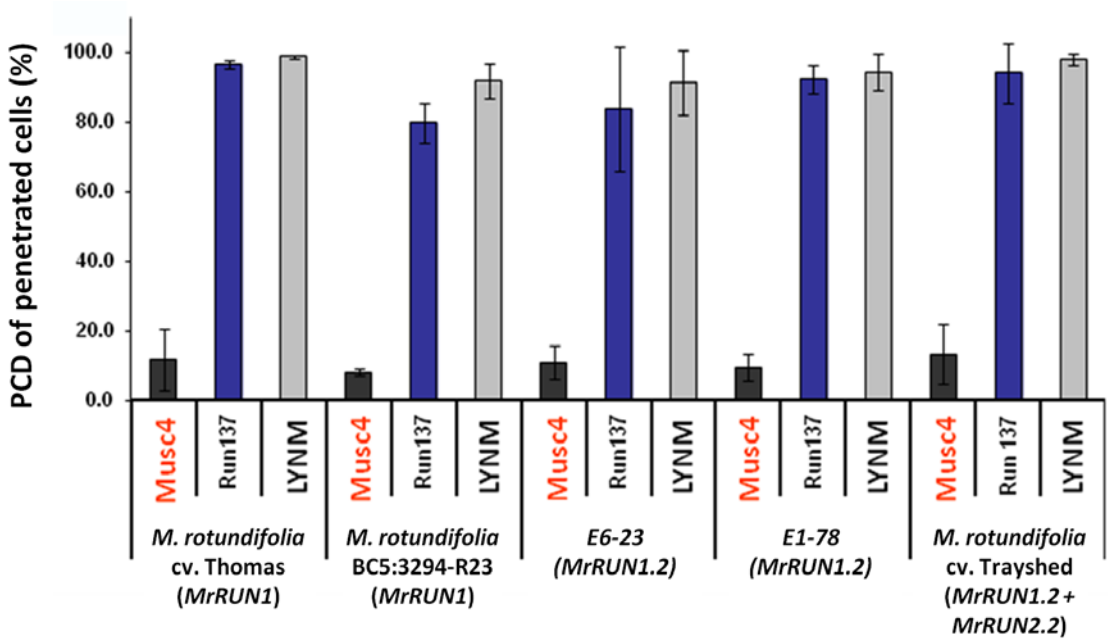


Figure 15. *Induction of programmed cell death (PCD) in penetrated epidermal cells of Muscadinia rotundifolia cultivars or introgression lines, 48 hpi with the Erysiphe necator isolates LNYM, Run137, or Musc4. BC5:3294-R23 is derived from M. rotundifolia cv. Thomas while breeding lines E6-23 and E1-78 are derived from M. rotundifolia cv. Trayshed. At least 100 germinated spores were scored for PCD. Each data point is the mean ±SE of three independent experiments where two leaves were scored.*

Vitis candicans as a potential source of powdery mildew penetration resistance

Analysis of the North American wild Vitis species *V. candicans* (also known as *V. mustangensis*) demonstrated a high level of resistance to powdery mildew infection and that the resistance appeared to be expressed predominately at the penetration phase of infection rather than post penetration through the induction of PCD as in the case of *MrRUN1* (Figure 16). Up to 65% of attempted penetrations of epidermal cells of *V. candicans* failed, in comparison to <20% on a normal *V. vinifera* susceptible cultivar such as Shiraz (Figure 16). As this represents a completely different resistance strategy against powdery mildew infection to that observed in grapevines containing *MrRUN1,* the gene(s) controlling this enhanced penetration resistance represents an attractive target for pyramiding with *MrRUN1* resistance.

To investigate *V. candicans* resistance further, we undertook a cross of *V. candicans* with the powdery mildew susceptible cultivar Cabernet Sauvignon. A segregating population of 37 individuals was established in the glasshouse and screened for the inheritance of penetration resistance. Penetration resistance segregated as a quantitative trait (Figure 17) ranging from levels equivalent to that observed in the susceptible Cabernet Sauvignon parent up to levels observed in *V. candicans*. These results indicate that powdery mildew penetration resistance from *V. candicans* involves the action of at least two independently segregating loci and was therefore not pursued any further.

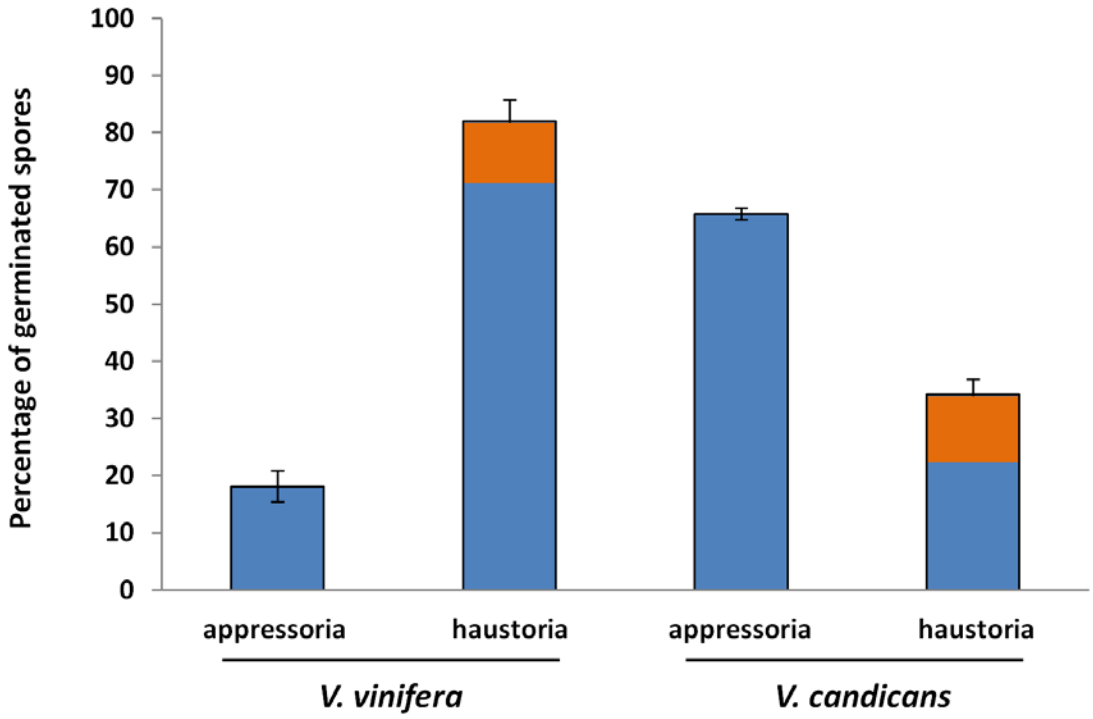


Figure 16. *Comparison of penetration resistance of V. vinifera cv. Shiraz and V. candicans to E. necator infection. Data shows the outcome of E. necator infection on leaves 48 h post-inoculation. Results are expressed as the percentage of germinated spores which produced an appressorium but did not penetrate or produced an appressorium and successful penetrated resulting in the formation of a haustorium. The percentage of cells containing a haustorium which had also undergone programmed cell death (PCD) is shown in orange. Each data point (mean ± SE) is based on two leaves and a minimum of 100 germinated conidia per leaf.*

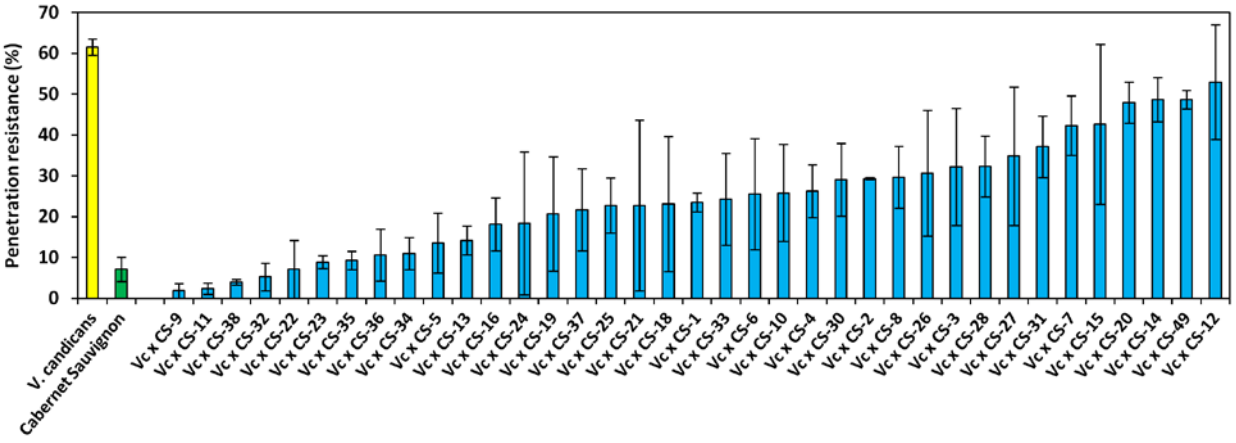


Figure 17. *Screening of powdery mildew penetration resistance in F1 progeny of a V. candicans x V. vinifera cv. Cabernet Sauvignon cross. Data shows the outcome of E. necator infection on leaves 48 h post-inoculation. Penetration resistance was assessed by scoring whether the fungus had successfully penetrated the epidermal cell as indicated by the presence of a visible haustorium or the production of secondary hyphae. Each data point (mean ± SE) is based on at least two leaves sampled on different days and a minimum of 100 germinated conidia per leaf.*

Investigation of Vitis cinerea as a potential source of downy mildew resistance

This section of the final report is commercial in confidence and will not be available until after June 2015. If you have any questions please contact the GWRDC

Investigation of wild Chinese Vitis species as a source of powdery mildew resistance

This section of the final report is commercial in confidence and will not be available until after June 2015. If you have any questions please contact the GWRDC

Evaluation of strategies for simultaneous transformation with multiple resistance genes

In order to be able to pyramid multiple *R* genes in new transgenic grapevines we need to be able to introduce more than one *R* gene at a time. We chose to investigate the feasibility of this approach by attempting to introduce the *MrRUN1* powdery mildew gene and the *MrRPV1* downy mildew gene into transgenic grapevines simultaneously.

Two different strategies were tested. Strategy A involved cloning *MrRUN1* and *MrRPV1* into the same binary transformation vector and then carrying out grapevine transformation and selection as normal. However, due to the size constraints on cloning large genomic DNA fragments into binary transformation vectors it was not possible to clone the large genomic DNA fragments containing *MrRUN1* (*MrRGA-10*; 11.5kb) and *MrRPV1* (*MrRGA-8*; 14.7 kb) into the same binary vector. Therefore a decision was made to use the cDNA versions of the coding regions of each gene to significantly reduce the size of the fragment to be introduced into the vector, while maintaining the same promoter region. This reduced the size of the inserts from 11.5 kb to 5.4 kb in the case of MrRUN1 and 14.7 kb to 5.9 kb for MrRPV1 enabling us to combine them both in a single transformation vector designated pCLB2301-RGA8 cDNA + RGA10 cDNA. As we had not previously tested transgenics transformed with the cDNA versions of *MrRGA10* (*MrRUN1)* and *MrRGA8* (*MrRPV1*), it was also necessary to generate transformation constructs with the two genes individually and these were designated pCLB2301-RGA8 cDNA and pCLB2301NK-RGA10 cDNA. All three constructs were transformed into *V. vinifera* cv. Shiraz embryogenic callus and transgenic lines selected as reported previously. Putative transgenic lines were confirmed to be expressing the introduced transgenes by qPCR.

As this objective was only commenced in the final year of the project the number of transgenic lines generated has been limited and duplicate phenotyping assays have not yet been completed on all lines. In total, 8 MrRGA8 + MrRGA10 cDNA, 4 MrRGA8 cDNA and 15 MrRGA10 cDNA transgenics were successfully established in the glasshouse and phenotyped at least once for downy mildew and/or powdery resistance.

Surprisingly, inoculation of the MrRGA8 + MrRGA10 cDNA transgenics, with downy mildew, indicated that none of the dual cDNA transgenics showed any significant increase in resistance to this pathogen with all transgenics supporting levels of sporulation similar to the untransformed Shiraz control (data not shown). Powdery mildew resistance was assayed by measuring PCD per penetrated epidermal cell. Of the 8 lines tested, 6 did not appear to show any significant increase in powdery mildew induced PCD above that observed in the untransformed Shiraz control (~2-3 %; Figure 27a). The other 2 lines (8+10-2 and 8+10-3) showed minor increases in PCD (13-15 %) but the levels observed were still well below that typically observed with transgenic Shiraz lines transformed with a genomic RGA10 construct (i.e. 65-85 %; Figure 27a) and would not be sufficient to have any significant impact on powdery mildew development.

To determine whether this was a problem related to the dual R gene construct or to the use of the cDNA coding region instead of the genomic region, we analysed downy mildew and powdery mildew resistance levels in the single cDNA transgenics. Again, none of the 4 MrRGA8 cDNA transgenics displayed any increase in resistance to downy mildew (data not shown). Testing of the MrRGA10 cDNA transgenics with powdery mildew gave similar results to that observed with the MrRGA8 + MrRGA10 cDNA transgenics. Twelve out of fifteen did not appear to show any significant increase in powdery mildew induced PCD above that

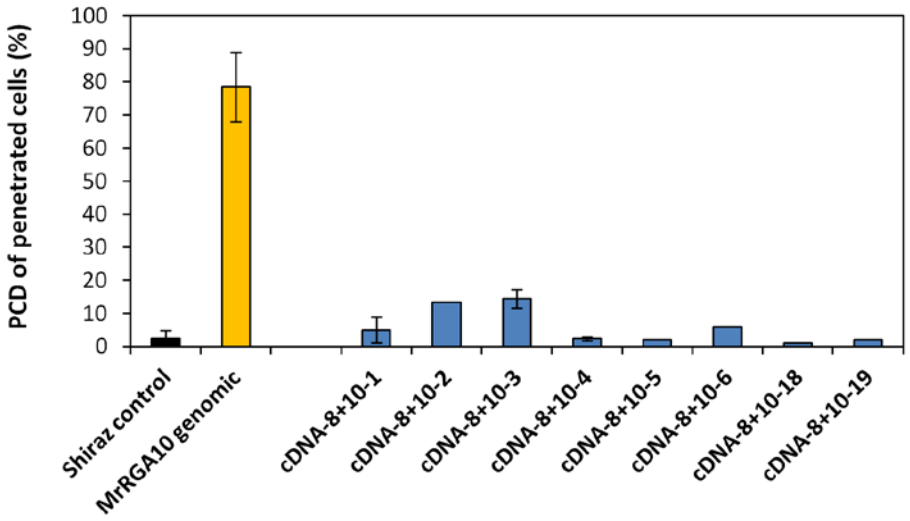
observed in the untransformed Shiraz control (Figure 27b) and the other 3 showed only a minor increase in PCD induction (9-14 %).

The results indicate that, although we have been successful in generating transgenic grapevines expressing dual *R* gene constructs, for reasons that are not yet clear, it would appear that the introduction of the MrRGA8/10 cDNA coding region instead of the full MrRGA8/10 genomic region, does not result in the generation of grapevines with increased resistance to powdery or downy mildew. One possibility is that the level of mRNA expression from the cDNA construct is much lower than from the genomic construct, due to the influence of intron-mediated enhancement of transcription which has been observed for many plant genes (Parra *et al*., 2011). If this turns out to be the case for *MrRUN1* (*MrRGA10*) and *MrRPV1* (*MrRGA8*) and other *R* genes to be cloned in the future, then further work will be required to identify which of the introns within these genes are contributing to the transcriptional enhancement and make sure these are included in any future constructs based on the cDNA coding region.

The second strategy that was investigated to simultaneously introduce dual *R* genes into the same grapevine cells was based on the use of mixed Agrobacterium cultures at the co-cultivation stage and the use of dual selectable markers to identify cells which had been transformed with T-DNA from two different vectors carrying different *R* genes (Figure 28). Such an approach has been shown to work for tobacco (McKnight *et al*., 1987), rapeseed (DeBlock *et al*., 1991) and maize (Miller *et al*., 2002) but no data are currently available on the feasibility of this approach for grapevine transformation.

Transformation of grapevine embryogenic cultures with dual Agrobacterium cultures was carried out in essentially the same as for single Agrobacterium cultures except that two Agrobacterium cultures were mixed in equal proportions immediately prior to the co-cultivation step. Each Agrobacterium culture contained a different binary transformation vector with a different selectable marker and either *MrRGA8* (*MrRPV1*) or *MrRGA10* (*MrRUN1*) (Figure 28). The two different selectable markers used in this experiment were the GFP reporter gene and the kanamycin resistance gene. Transformed callus was incubated on kanamycin to select for embryos that had been transformed with the T-DNA containing MrRGA10 (vector 2; Figure 28). Kanamycin-resistant embryos were then visually screened under a fluorescent microscope for the presence of expressed green fluorescent protein (GFP; vector 1) which was linked to presence of *MrRGA8*. Embryos that satisfied both selection criteria were selected for further propagation and finally transferred to the glasshouse for phenotyping.

(a)



(b)

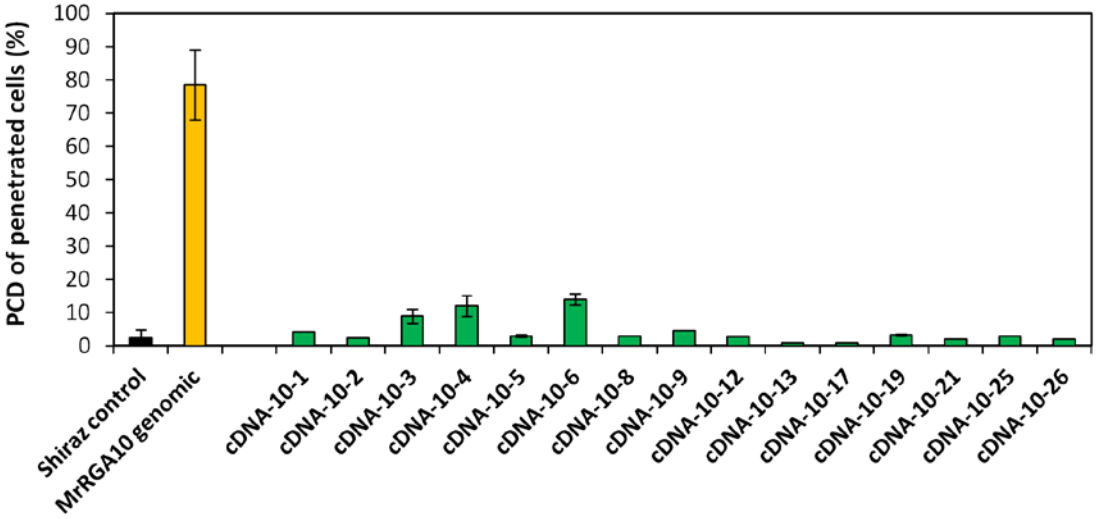


Figure 27. *Screening transgenics for powdery mildew-induced PCD in penetrated epidermal cells. a) Shiraz transgenic lines (cDNA-8+10) transformed with the dual MrRGA8 cDNA + MrRGA10 cDNA construct (pCLB2301-RGA8 cDNA + RGA10 cDNA). b) Shiraz transgenic lines (cDNA-10) transformed with a MrRGA10 cDNA construct only (pCLB2301-RGA8 cDNA + RGA10 cDNA). Leaves were sampled 48 hpi and scored for the induction of PCD using trypan blue staining. Data for untransformed Shiraz control and transgenic Shiraz transformed with a genomic RGA10 construct have been included for comparison. At least 100 germinated spores were scored for PCD.*

Twenty nine transgenics were generated that were confirmed by PCR to contain at least a portion of both the RGA8 and RGA10 genomic constructs. Of these 17/29 were found to have significantly increased levels of powdery mildew-induced PCD in penetrated epidermal cells ranging from 40-90% (Figure 29a). Of these 7/17 were found to also show significantly reduced levels of downy mildew sporulation comparable to that observed in the parental BC5:3294-R23 line containing the entire MrRUN1/MrRPV1 locus (Figure 29b).

These results confirm that the dual Agrobacterium system is a relatively simple and efficient modification to the existing grapevine transformation protocol to enable the introduction of at least two *R* genes simultaneously into grapevine cells and will facilitate the pyramiding of multiple *R* genes within the same transgenic grapevine.

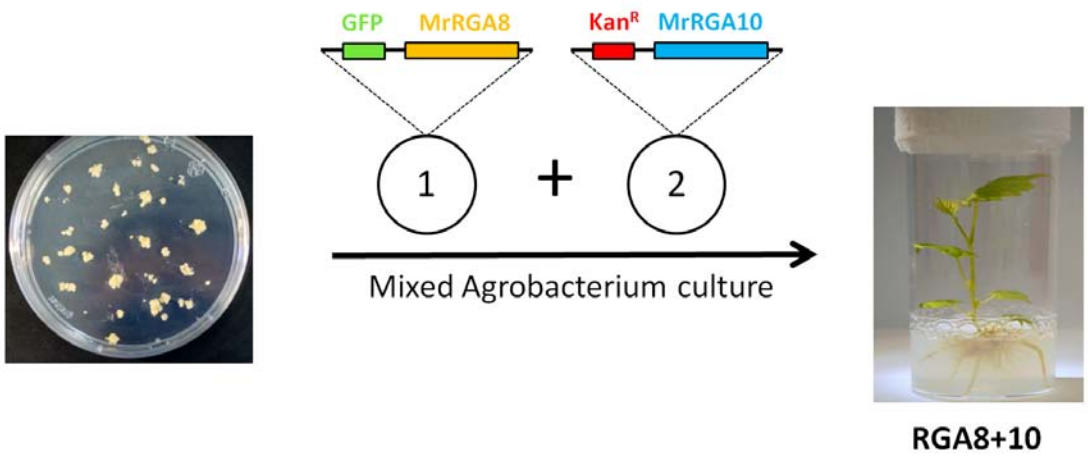


Figure 28. *Strategy for simultaneous transformation of embryogenic grapevine callus with MrRGA8 (MrRPV1) and MrRGA10 (MrRUN1) using a mixture of two Agrobacterium cultures containing different binary transformation vectors with different selectable markers.*

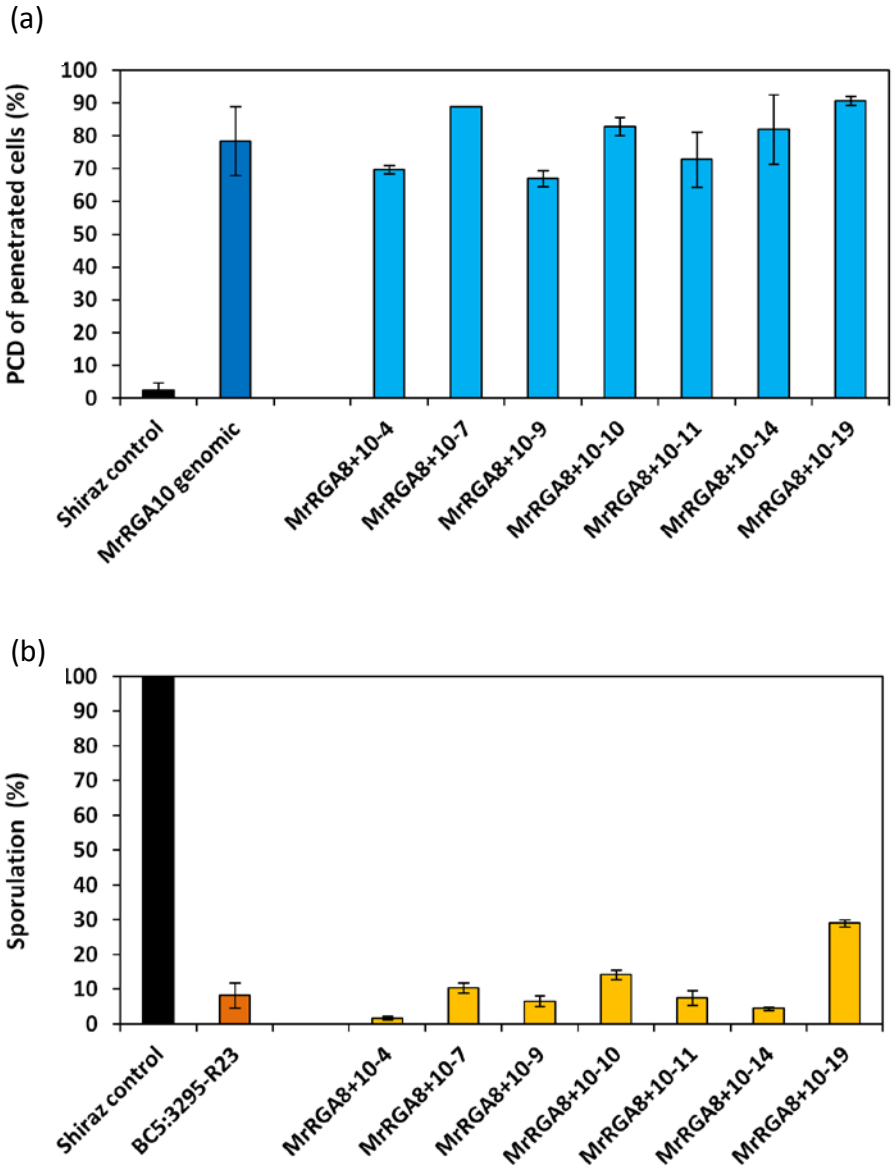


Figure 29. *Screening transgenics generated using the dual Agrobacterium transformation technique for powdery mildew and downy mildew resistance. a) Powdery mildew induced PCD in penetrated epidermal cells. Leaves were sampled 48 hpi and scored for the induction of PCD using trypan blue staining. Untransformed Shiraz control and transgenic Shiraz transformed with a genomic RGA10 construct were also included for comparison. At least 100 germinated spores were scored for PCD. b) Sporulation of leaf discs 6 dpi expressed relative to untransformed Shiraz control leaves. Five leaf discs were sampled from 2 individual leaves. The parental line BC5:3294-R23 containing MrRPV1 was also included for comparison*

This page intentionally left blank

8. The role of *VvMLO* genes in powdery mildew susceptibility

Introduction

In the previous GWRDC project CSP 0604 we reported on the cloning and expression analysis of 17 members of the grapevine *MLO* gene family. Based on a comparison of the predicted sequences of all members of the VvMLO family with MLO proteins from Arabidopsis and tomato that had been shown to have a role in powdery mildew susceptibility (Consonni *et al.,* 2006; Bai *et al.*, 2008), we postulated that *VvMLO* genes 3, 4, 13 & 17 were possible candidates for fulfilling a similar role in grapevine. Since that report was produced, a number of other MLO proteins have been identified from *Pisum sativum* (Humphry *et al.*, 2011), *Capsicum annuum* (Zheng *et al*., 2013), *Medicago truncatula* and *Lotus japonica* (personal communication, Dr Ralph Panstruga) that also appear to have a role in powdery mildew susceptibility in these species. A revised phylogenetic analysis including these new sequences (Figure 30) confirms the relatedness of MLO proteins involved in powdery mildew susceptibility and *VvMLO* genes 3, 4, 13 & 17 as potential candidates for this role in grapevine.

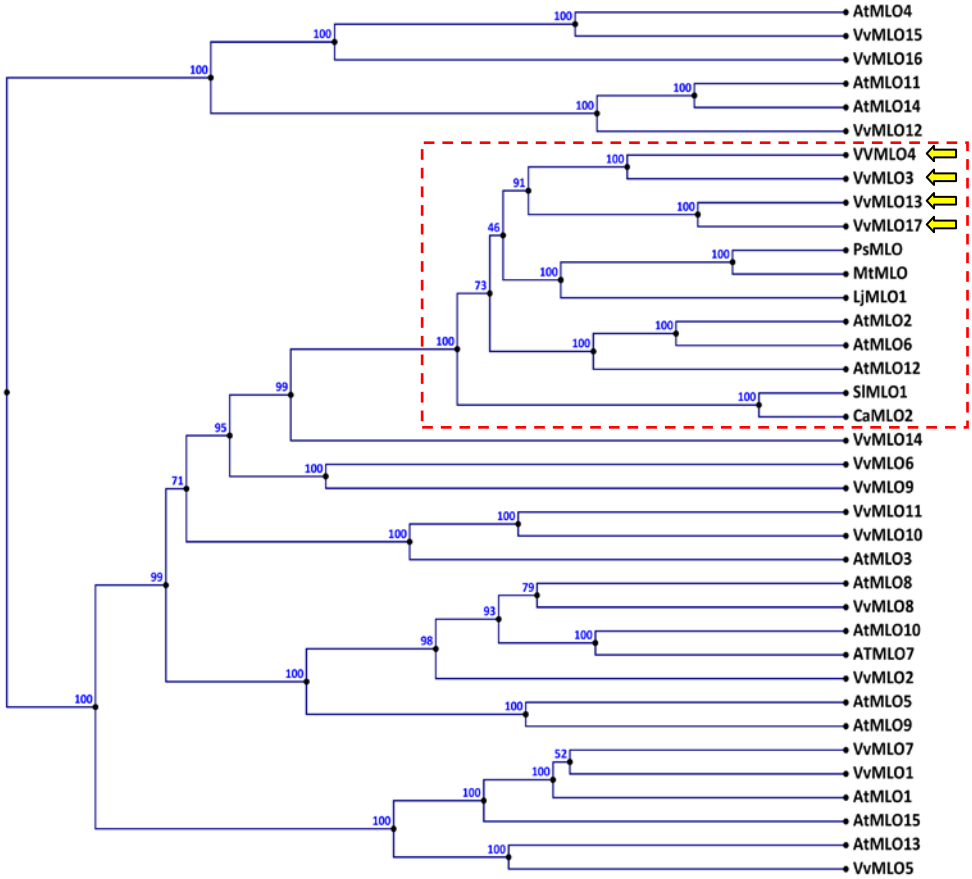


Figure 30. *Phylogenetic relationship of MLO isoforms. A UPGMA phylogenetic tree of the amino acid sequences of MLO family members of V. vinifera (VvMLO), A. thaliana (AtMLO) and selected proteins from tomato (SlMLO1), P. sativum (PsMLO), C. annuum (CaMLO2), M. truncatula (MtMLO) and L. japonica (LjMLO1). Numbers indicate bootstrap values (out of 100 replicates) that support the respective branch. The red box highlights a cluster of MLO proteins that have a role in powdery mildew susceptibility. The yellow arrows highlight grape MLO proteins in this cluster*

In the previous GWRDC project CSP 0604 we also presented data on the transcriptional responses of *VvMLO* genes to powdery mildew infection and based on this data postulated the most likely candidates to be *VvMLO* genes 3, 4 & 17. Subsequently, gene-silencing constructs utilising hairpin (hp) RNAi designed to *VvMLO3*, *VvMLO4* and *VvMLO17* were transformed into the *V. vinifera* microvine to determine whether these genes have any role in powdery mildew susceptibility. This work has continued in this current project and we report on progress below.

Materials and Methods

VvMLO constructs

Hairpin (hp) silencing constructs were designed to ~ 280 bp fragments in the 3' region of the target genes *VvMLO3*, *4*, *13* & *17* using the primer pairs: VvMLO3-hp1-F/R, VvMLO4-hp1-F/R, VvMLO13-hp1-F/R and VvMLO17-hp1-F/R. The region selected had the least sequence homology to the other *VvMLO* genes to avoid silencing of other members of the *VvMLO* gene family. Hairpin fragments were amplified by PCR, cloned into pDONR221 (Invitrogen) and then transferred into the CSIRO destination vector pHellsgate12 by Gateway cloning technology. The final cloning step for the *VvMLO* silencing constructs involved transfer of the silencing cassette into the pCLB1301NH binary vector for grapevine transformation.

To make the double (hpVvMLO3/4) and triple (hpVvMLO3/4/17) silencing constructs the same ~280bp regions were used as for the single knockouts described above. To generate hpVvMLO3/4, The hp*VvMLO3* region was first amplified with primers VvMLO3-hp1-F/R (without gateway adapters) by *Pfu* Ultra II Fusion HS DNA polymerase (Stratagene) and cloned into pCR Blunt (Life Technologies). The hp*VvMLO4* region was amplified with primers VvMLO4-hp1-F/R (without gateway adapters) using Platinum HiFi DNA polymerase (Life Technologies) and cloned into pDrive (Qiagen). To combine the two hp fragments into the same vector, a *Kpn* I/*Pst* I digest of pCR-Blunt hpVvMLO3 was made releasing the ~300 bp fragment which was cloned into the *Kpn* I/*Pst* I site of pDrive-hpVvMlo4 to make pDrive-hpVvMLO3/4. This was then used as a template for amplifying the double knockout fragment (~650bp) with primers VvMLO3-hp1-F and VvMLO4-hp1-R, cloned into gateway vector pDONR221 and then transferred into the destination vector pHellsgate12.

The triple knockout (hpVvMLO3/4/17) was made by first generating two PCR products and utilising a PCR fusion cloning technique. The hpVvMlo3-4 fragment (~650bp) was amplified with the primer pair VvMLO3-hp1-F and VvMLO4-hp1-R (without gateway adapters). A forward primer containing 30bp of sequence identical to the 3' end of hpVvMLO4 and the rest to VvMLO17 (VvMLO4-17 F1) together with VvMLO17-hp1-R was used to amplify hpVvMLO17 (~350 bp). For PCR fragment fusion the following 30ul reaction mixture was set up: 200-800ng of each PCR product (equal volume of each fragment was used), 3ul of 10x *Pfu* Ultra II reaction buffer, 3ul of 2mM dNTP mix and 0.3ul of *Pfu* Ultra II Fusion HS DNA Polymerase. The mixture was then placed into a thermocycler at 98°C for 30 sec, 60°C for 1 min and 72°C for 7 min. The fused fragment (~950bp) was then gel-purified, cloned into pDONR221 and transferred into the destination vector pHellsgate12 by gateway cloning technology. As with the single hp constructs, the final cloning step involved transferring the double hp or triple hp cassette into the binary vector pCLB1301NH for grapevine transformation.

Analysis of endogenous VvMLO gene expression in hpVvMLO transgenics
Leaves were collected from transgenic Shiraz plants transformed with the following silencing constructs: hpVvMLO3, hpVvMLO4, hpVvMLO13, hpVvMLO17, hpVvMLO3/4 and hpVvMLO3/4/17 and used for qRT-PCR analysis as described on Page 16.

Assessment of powdery mildew susceptibility

Susceptibility to powdery mildew penetration (penetration efficiency) was assessed by determining the percentage of germinated powdery mildew spores that successfully penetrated the epidermal cell below the appressoria as indicated by the presence of a visible haustorium or the production of secondary hyphae. A minimum of 100 germinated conidia were scored per leaf. In Shiraz, typically 80-85% of attempted penetrations are successful. *MLO*-mediated resistance in other plant species leads to a significant decrease in the percentage of successful penetrations (or increased resistance to penetration).

Results and Discussion

Single hpVvMLO silencing constructs transformed in V. vinifera microvine

As outlined in GWRDC final project report CSP 0604, progress on evaluation of powdery mildew resistance in transgenic microvines containing *hpVvMLO* silencing constructs had been severely hampered by two technical issues: (a) hairiness of the leaves and (b) high background levels of penetration resistance in untransformed microvines. This made it very difficult to interpret the results obtained with the transgenic microvines.

In an effort to overcome these issues it was proposed that we allow the hpVvMLO transgenic microvines to undergo self-fertilization, set seed and then germinate the seed under conditions which specifically select for segregating normal “tall” or non-dwarf progeny vines to avoid some of the confounding factors associated with the microvine dwarf phenotype. There is also the added advantage that one third of the non-dwarf progeny vines should be homozygous for the hpVvMLO silencing construct which may lead to higher levels of *VvMLO* suppression and possibly reduced powdery mildew susceptibility.

Of the 27 transgenic microvine lines containing hpVvMLO silencing constructs, 20 produced fruit from which seed was collected. Unfortunately, efficiency of seed germination proved to be quite low and we were only able to establish segregants (ranging from 1-6 per line) of 16 individual microvine transgenic lines (6 hpVvMLO3, 2 hpVvMLO4, 8 hpVvMLO17) in the glasshouse for powdery mildew phenotyping. The non-dwarf vines selected were checked for the presence/absence of the hpVvMLO *s*ilencing construct by genomic PCR, inoculated with powdery mildew and penetration rates compared to untransformed Shiraz vines.

A number of non-dwarf lines transformed with either *hpVvMLO3*, *hpVvMLO4* or *hpVvMLO17* were identified to have significantly reduced levels of powdery mildew penetration compared to untransformed Shiraz control vines (Figure 31a). However, when selected vines were examined for endogenous levels of *VvMLO3*, *VvMLO4* and *VvMLO17* transcription, the reduced level of powdery mildew penetration was not found to be correlated with a reduced expression of the endogenous *VvMLO3*, *VvMLO4* or *VvMLO17* genes in these lines (Figure 31b). Thus, the observed reduction in powdery mildew susceptibility could not be attributed to a silencing of these genes and must have been due to other genetic factors arising from transformation and segregation. Based on these results a decision was taken to halt any

further analysis of these microvine-derived hpVvMLO transgenics and focus on the direct transformation of hpVvMLO silencing constructs into Shiraz.

Multiple hpVvMLO silencing constructs transformed in V. vinifera cv. Shiraz

Results of studies on *MLO*-mediated powdery mildew susceptibility in Arabidopsis had previously demonstrated that full resistance was only achieved when all three closely related genes i.e. *AtMLO2*, *AtMLO6* and *AtMLO12* were silenced suggesting a level of redundancy between these different family members (Consonni *et al.,* 2006). As grapevine also appears to have three closely-related MLO genes (*VvMLO3*, *VvMLO4* & *VvMLO17*) that are good candidates to be involved in powdery mildew susceptibility, a decision was made to target all three *VvMLO* genes simultaneously with a triple hp RNAi silencing construct (hpVvMLO3/4/17). Based on complementation studies reported first in the GWRDC final project report CSP 0604 and subsequently in Feechan *et al.* (2013), which confirmed *VvMLO3* and *VvMLO4* to be powdery mildew susceptibility genes based complentation assays in the Arabidopsis *Atmlo2 mlo6 mlo12* triple mutant, a second double hp silencing construct (hpVvMLO3/4) was also transformed into Shiraz embryogenic callus.

A total of 21 hpVvMLO3/4/17 and 25 hpVvMLO3/4 Shiraz transgenics lines were generated and transferred to the glasshouse for evaluation of powdery mildew susceptibility and endogenous *VvMLO* expression. The majority of transgenic lines analysed did not show any significant reduction in powdery mildew penetration compared to untransformed Shiraz control vines, with a few lines containing either the double or triple hpVvMLO constructs showing a 15-25 % reduction in powdery mildew penetration (Figure 32). Analysis of endogenous *VvMLO* expression in these lines was again inconclusive with no apparent correlation between powdery mildew susceptibility and the endogenous expression of *VvMLO3*, *VvMLO4* or *VvMLO17* (Figure 33).

Furthermore, based on the number of transgenics generated containing these hpVvMLO RNAi silencing constructs we would have expected to find a number of transgenic lines in which the level of endogenous *VvMLO3*, *VvMLO4* or *VvMLO17* transcription was significantly reduced. For example, the Flavonoid research group in the CSIRO PI Adelaide laboratory has successfully utilised hp RNAi silencing constructs to modify flavonoid pathway genes in grapevines and their results indicate that most of their hp RNAi transgenics show levels of endogenous gene expression of 20-50 % (compared to wild-type plants) with some as low as 8-10% (personal communication, Dr Mandy Walker). However, the transgenics recovered from our hpVvMLO RNAi experiment show, at best, only minor reductions in the transcription of the targeted VvMLO genes (Figure 33). This led us to speculate that these triple and double RNAi silencing constructs may be lethal in our grapevine transformation system, such that plants in which multiple VvMLO genes have been severely silenced cannot be recovered.

To investigate this possibility further we carried out another series of Shiraz transformations in which we used the single hpVvMLO silencing constructs, as used previously with the microvine transformation.

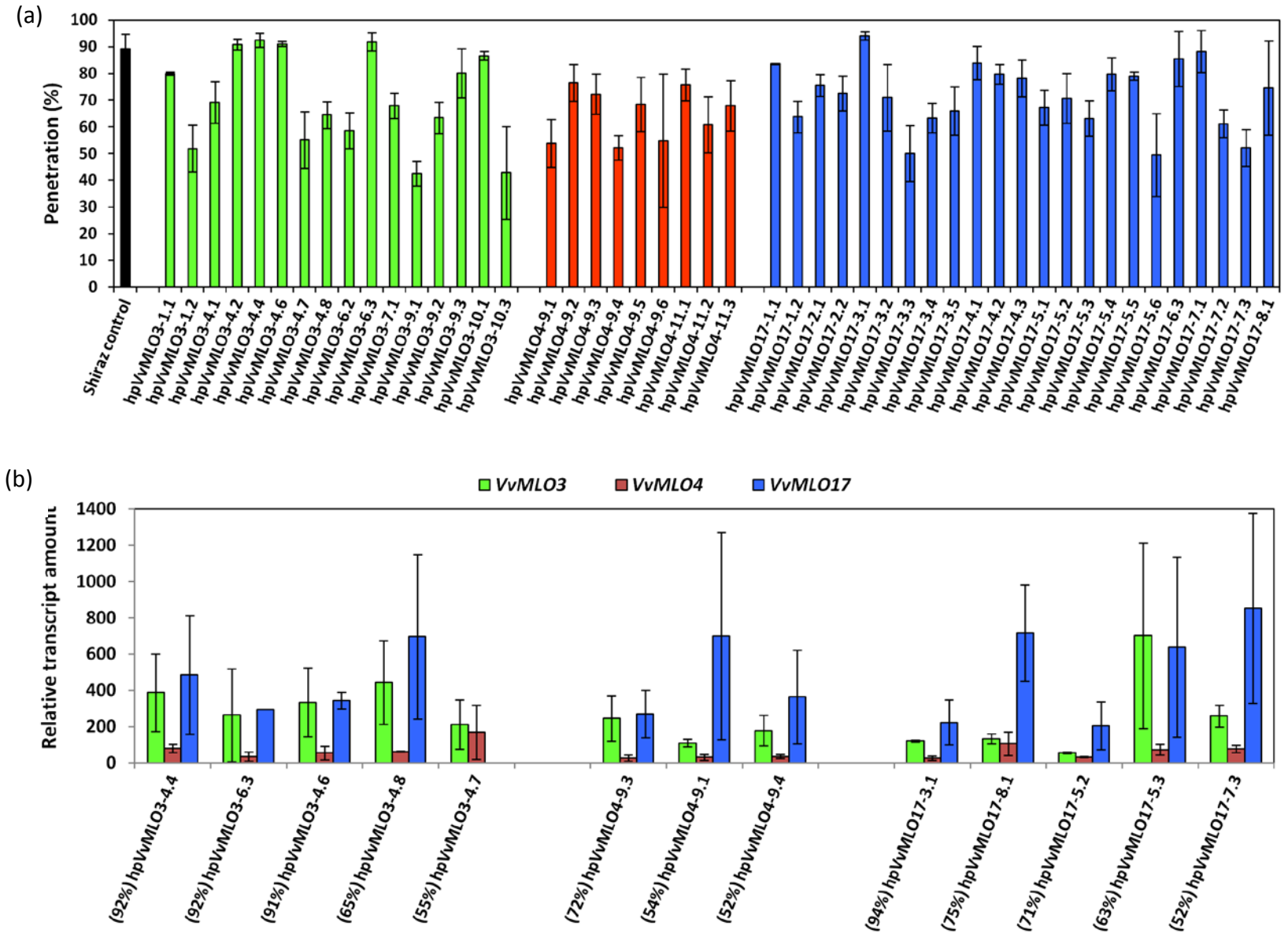


Figure 31. *Screening of transgenic microvine lines containing single hpVvMLO silencing constructs. (a) Penetration efficiency measured 48 h post-inoculation. (b) endogenous VvMLO gene expression. Each data point (mean ± SE) is based on at least two leaves sampled on different days.*

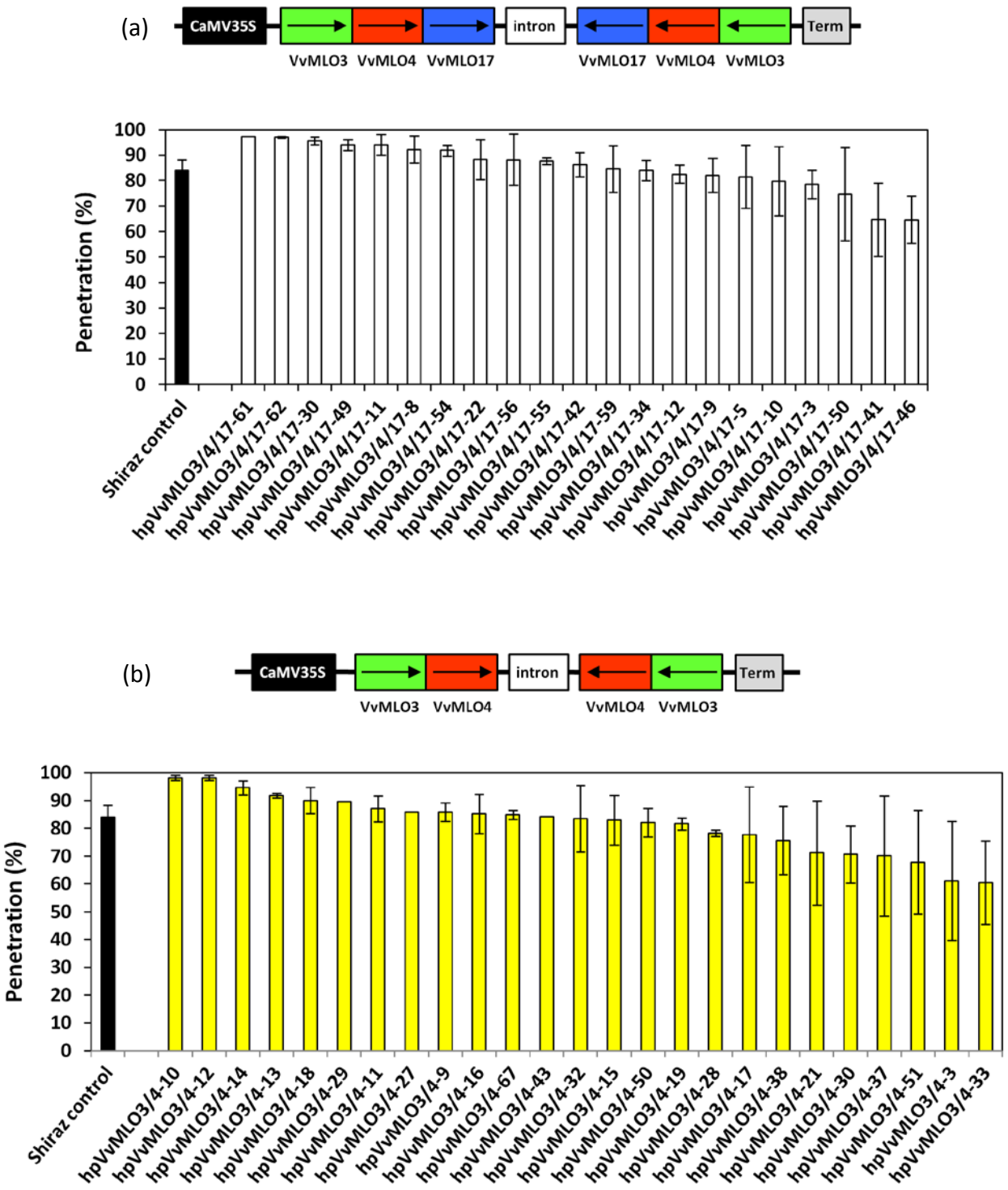


Figure 32. *Screening of transgenic Shiraz lines containing triple (a) and double (b) hpVvMLO silencing constructs. Penetration efficiency measured 48 h post-inoculation. Each data point (mean ± SE) is based on at least two leaves sampled on different days.*

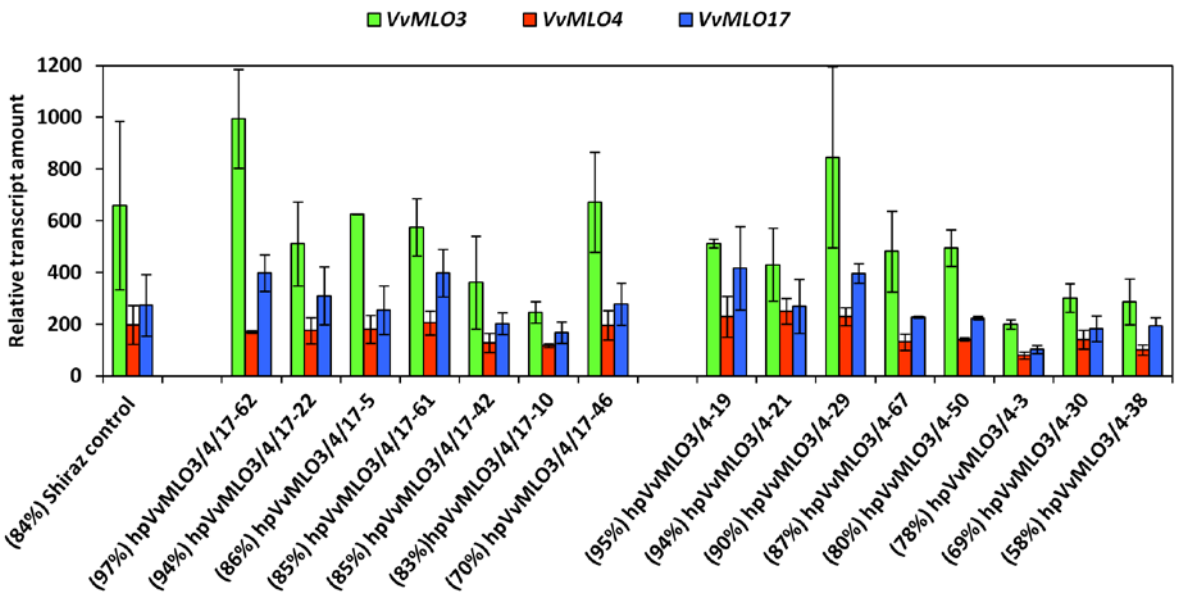


Figure 33. *Screening of endogenous VvMLO gene expression in transgenic Shiraz lines containing triple (3/4/17) and double (3/4) hpVvMLO silencing constructs. Transcript levels of VvMLO3 (green), VvMLO4 (red) and VvMLO17 (blue) were measured by qPCR. Each data point (mean ± SE) is based on at least two leaves sampled on different days.*

Single hpVvMLO silencing constructs transformed into V. vinifera cv. Shiraz

This section of the final report is commercial in confidence and will not be available until after June 2015. If you have any questions please contact the GWRDC.

This page intentionally left blank

9. Identification of putative *Erysiphe necator* effectors secreted during infection

This section of the final report is commercial in confidence and will not be available until after June 2015. If you have any questions please contact the GWRDC.

This page intentionally left blank

10. Outcomes / Conclusions

The major outcomes/conclusions of this project can be summarised as follows:

- The *MrRUN1* gene from *M. rotundifolia* confers complete resistance to powdery mildew isolates in Australia, Europe and north-eastern USA but is broken by isolates collected from south-eastern USA from where Muscadine vines originate.

- The *MrRPV1* gene from *M. rotundifolia* confers strong resistance to downy mildew isolates in Australia, Europe and North America.

- The *MrRUN1/MrRPV1* locus has been successfully introgressed into premium winegrape varieties by classical hybridization and appear to provide good resistance to powdery and downy mildew in unsprayed blocks in the Barossa Valley. Some grape genotypes appear to have a minor suppressive effect on resistance conferred by *MrRUN1 and MrRPV1* but this is unlikely to reduce their effectiveness under growing conditions in most Australian vineyards.

- *Vitis candicans* demonstrated a high level of resistance to powdery mildew that appeared to operate predominately at the penetration phase of infection rather than through the induction of PCD following penetration as observed with *MrRUN1.* Unfortunately this penetration-based resistance appears to involve the action of at least two independently segregating loci making it difficult to characterise any further.

- *Vitis cinerea* contains a major QTL conferring downy mildew resistance that has been successfully introgressed into the *V. vinifera* microvine.

- The Chinese wild Vitis species, *V. romanetii* and *V. piasezkii*, both show complete resistance to powdery mildew. While we currently have no information as to the number of genes conferring resistance in *V. piasezkii*, published data from USDA researchers indicates that powdery mildew resistance in *V. romanetii* is conferred by a single dominant gene designated REN4. Results of other studies carried out in collaboration with these USDA researchers confirms that *REN4* would be an excellent candidate for pyramiding with *MrRUN1* because it would extend the range of isolates to which these vines would be resistant and increase the durability of resistance in the vineyard. To facilitate this goal, we have successfully imported seed of a cross, carried out in the USA, between C87-14 (BC1-*REN4* resistant parent) x *V. vinifera* cv. White Riesling, under a Material Transfer Agreement, that gives CSIRO and GWRDC freedom to operate. Progeny derived from this seed has been confirmed by CSIRO PI to be segregating for *REN4* resistance.

- We have successfully demonstrated for the first time that two *R* genes can be simultaneously introduced into grapevine cells using the dual Agrobacterium system. This will facilitate the pyramiding of multiple *R* genes within the same transgenic grapevine.

- Next Generation sequencing technology has been used to successfully identify putative effector proteins secreted by grapevine powdery mildew during infection. This opens up the possibility of identifying the specific avirulence effector recognised by MrRUN1 and then designing a genetic screen to enable us to track the potential appearance of *MrRUN1*-breaking powdery mildew strains in the vineyard.

This page intentionally left blank

11. Recommendations

- The successful introduction of powdery and downy mildew resistance genes from the wild North American species *M. rotundifolia* into commercial *V. vinifera* cultivars by genetic transformation offers the possibility of growing existing premium winegrape cultivars with significantly reduced agrochemical and carbon inputs. However, until there is a significant shift in public perception, especially in European markets, regarding the acceptance of wine produced from transgenic scions, there appears to be no immediate possibility of deploying these mildew resistant vines in Australian vineyards. Nonetheless, it will be crucial for the Australian Grape & Wine Industry to continue to invest in grapevine transformation capability in addition to supporting germplasm improvement programs through MAS. Transgenic approaches offer the potential to make rapid step-changes in existing premium varieties in terms of adaptability to future major challenges of climate change, disease control and sustainability and would place the Australian industry is in a position to swiftly capitalize on any changes in market acceptance of wines made from transgenic vines.
- New disease-resistant germplasm containing the *MrRUN1/MrRPV1* locus, generated by MAS, should be assessed for disease-resistance in unsprayed blocks in a range of different vineyard sites throughout Australia.
- New sources of genetic resistance to powdery and downy mildew from the North American species *V. cinerea* and the Chinese species *V. amurensis* and *V. romanetii* need to be introgressed into *V. vinifera* varieties for pyramiding with *MrRUN1* and *MrRPV1*. Genetic markers closely linked to these disease resistance loci also need to be developed to enable selection of progeny containing multiple resistance genes.
- Genetic markers for other desirable traits such as open bunch architecture (for reduced susceptibility to Botrytis bunch rot), heat tolerance, drought resistance and specific grape flavour compounds should also be developed to be used in combination with existing markers for powdery and downy mildew resistance, berry colour, and flower sex for MAS of enhanced disease-resistant wine varieties.
- Further bioinformatic analysis and functional testing of putative powdery mildew effectors (EnCSEPs) in virulent and avirulent powdery mildew strains is required to identify the avirulence effector recognised by MrRUN1 to enable a diagnostic assay to be developed for investigating the distribution of MrRUN1-breaking isolates.

This page intentionally left blank

Appendix 1: Communication

Scientific Publications

Moyer MM, Gadoury DM, Cadle-Davidson L, Dry IB, Wilcox WF, Seem RC (2010) Effects of acute low temperature events on development of *Erysiphe necator* and susceptibility of *Vitis vinifera.* *Phytopathology* **100**: 1240-1249.

Feechan A, Kabbara S, Dry IB (2011) Mechanisms of powdery mildew resistance in the Vitaceae family. *Molecular Plant Pathology* **12**: 263-274

Anderson C, Choisne N, Adam-Blondon A-F, Dry IB (2011) Positional cloning of disease resistance genes in grapevine. In: *Encyclopaedia of Plant Genomics: Grapes* (ed. C. Kolen) pp. 186-210. CRC Press.

Gadoury DM, Cadle-Davidson L, Wilcox WF, Dry IB, Seem RC, Milgroom MG (2012) Grapevine Powdery Mildew (*Erysiphe necator*): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. *Molecular Plant Pathology* **13**: 1-16.

Gadoury DM, Wakefield LM, Cadle-Davidson L, Dry IB, Seem RC (2012) Effects of prior vegetative growth, inoculum density, light, and mating on conidiation of *Erysiphe necator*. *Phytopathology* **102**: 65-72

Ayliffe M, Periyannan SK, Feechan A, Dry IB, Schumann U, Wang MB, Pryor A, Lagudah, E (2013) A simple method for comparing fungal biomass in infected plant tissues. *Molecular Plant Microbe Interactions* **26**: 658-667

Feechan A, Jermakow AM, Ivancevic A, Godfrey D, Pak H, Panstruga R, Dry IB (2013) Host cell entry of powdery mildew is correlated with endosomal transport of antagonistically acting VvPEN1 and VvMLO to the papilla. *Molecular Plant Microbe Interactions* **26**: 1138-1150

Feechan A, Anderson C, Torregrosa L, Jermakow A, Mestre P, Wiedemann-Merdinoglu S, Merdinoglu D, Walker AR, Cadle-Davidson L, Reisch B, Aubourg S, Bentahar N, Shrestha B, Bouquet A, Adam-Blondon A-F, Thomas MR, Dry IB (2013) Genetic dissection of a TIR-NB-LRR locus from the wild North American grapevine species *Muscadinia rotundifolia* identifies paralogous genes conferring resistance to major fungal and oomycete pathogens in cultivated grapevine**.** *Plant Journal* **76**: 661-674

Feechan A, Kabbara S, Dry IB (2014) Sources of penetration and PCD-mediated resistance to grapevine powdery mildew in the Vitaceae family. *Acta Horticulturae*, in press

Dry IB (2014) Recent progress in understanding the genetics of pest and disease resistance in grapevine. *Acta Horticulturae*, in press

Salami SA, Ebadi A, Naghavi MR, Dry IB (2014) Cloning and functional characterisation of a putative powdery mildew susceptibility gene in grapevine. *Acta Horticulturae*, in press

Conference Presentations

Moyer MM, Gadoury DM, Cadle-Davidson L, Dry IB, Magarey PA, Wilcox WF, Seem RC. "Acute low temperature events reduce the survival of *Erysiphe necator* and increase resistance in ordinarily-susceptible *Vitis vinifera* leaf tissue. 6th International Workshop of grapevine downy and powdery mildew, Bordeaux, France, 4-9th July, 2010. Poster

Gadoury DM, Wakefield LM, Seem RC, Cadle-Davidson L, Dry IB. "Effect of prior vegetative growth, inoculum density and light on conidiation in *Erysiphe necator.* 6th International Workshop of grapevine downy and powdery mildew, Bordeaux, France, 4-9th July, 2010. Poster

Dry IB. "Recent progress in understanding the genetics of pest and disease resistance in grapevine." 10th International Conference on Grapevine Breeding & Genetics, Geneva, New York, 1-5th August, 2010. Plenary talk

Feechan A, Kabbara S, Dry IB. "Sources of penetration and PCD-mediated resistance to grapevine powdery mildew in the Vitaceae family". 10th International Conference on Grapevine Breeding & Genetics, Geneva, New York, 1-5th August, 2010. Poster.

Salami SA, Ebadi A, Naghavi MR, Dry IB. "Cloning and functional characterisation of a putative powdery mildew susceptibility gene in grapevine." 10th International Conference on Grapevine Breeding & Genetics, Geneva, New York, 1-5th August, 2010. Poster.

Dry IB. "Biotechnology in the vineyard. New technologies for disease resistant varieties." AusBiotech conference, Adelaide, 16-19th October, 2011. Oral presentation

Dry IB, Feechan A, Thomas MR. "New vines for new times." 15th Australian Wine Industry Technical Conference. Sydney, NSW, 13-18th July, 2013. Oral presentation

Yin L, Feechan A, Lu J, Dry IB. "Function characterization of the grapevine *MrRPV1* downy mildew resistance gene." 10th International Congress of Plant Pathology, Beijing, China, 25-30th August, 2013. Poster

Dry IB. "Cloning and functional characterisation of a powdery mildew resistance gene from a wild North American grapevine." 3rd International Powdery Mildew Workshop, Copenhagen, Denmark, 29-30th August, 2013. Oral presentation

Industry Journal articles / Interviews

Dry IB, Jermakow A, Feechan A (2009) Do cultivated grapevines contain genes that predispose them to powdery mildew infection? A potential new strategy to develop powdery mildew resistant grapevines. *Australian & New Zealand Grapegrower & Winemaker* **549**: 46-50

Dry IB (2010) Grapevine genes for improved resistance to fungal pathogens to reduce chemical inputs in Australian vineyards. Australia *& New Zealand Grapegrower & Winemaker* **553**: GWRDC R&D at Work: Viticulture value-add p2.

Interviews were conducted and information provided for articles on the development of disease-resistant grapevine germplasm in:

- "Grapes Transformed" - *Wine Spectator*, December 2010
- "Growers blighted by white-out" - *Sunraysia Daily*, 22nd December 2010
- "Biotech arms crops for biowarfare" - *Australian Life Scientist,* April 2011
- "Australian scientist develops GM grapevines" - *ABC radio*, October 2011.
- "The next 'big thing' – genetic modification" - *Australian & New Zealand Grapegrower & Winemaker* December 2011.

Appendix 2: Intellectual Property

CSIRO obtained the *V. romanetii* BC2 population 12-3501 containing the *REN4* resistance locus from Dr David Ramming (USDA/ARS/CDPG, Parlier, California) under a USDA MTA agreement. CSIRO and GWRDC have complete freedom to use this material for the breeding of new disease-resistant winegrape varieties.

This page intentionally left blank

Appendix 3: References

Allen J and Salzberg SL (2005) JIGSAW: integration of multiple sources of evidence for gene prediction. *Bioinformatics* 21: 3596-3603

Bai Y, Pavan S, Zheng Z, Zappel NF, Reinstädler A, Lotti C, De Giovanni C, Ricciardi L, Lindhout P, Visser R, Theres K and Panstruga R (2008) Naturally occurring broad-spectrum powdery mildew resistance in a Central American tomato accession is caused by loss of *Mlo* function. *Mol. Plant Microbe Interact* 21: 30-39

Barker CL, Donald T, Pauquet J, Ratnaparkhe MB, Bouquet A, Adam-Blondon AF, Thomas MR and Dry IB (2005) Genetic and physical mapping of the grapevine powdery mildew resistance gene, *Run1*, using a bacterial artificial chromosome library. *Theor. Appl. Genet.* 111: 370-377.

Bernoux M, Ve T, Williams S, Warren C, Hatters D, Valkov E, Zhang X, Ellis JG, Kobe B, Dodds PN (2011) Structural and functional analysis of a plant resistance protein TIR domain reveals interfaces for self-association, signaling, and autoregulation. Cell Host Microbe 9: 200-11

Blanc S, Wiedemann-Merdinoglu S, Dumas V, Mestre P and Merdinoglu D (2012) A reference genetic map of *Muscadinia rotundifolia* and identification of *Ren5*, a new major locus for resistance to grapevine powdery mildew. *Theor. Appl. Genet.* 125: 1663-1675

Blasi P, Blanc S, Wiedemann-Merdinoglu S, Prado E, Ruhl EH, Mestre P and Merdinoglu D (2011) Construction of a reference linkage map of *Vitis amurensis* and genetic mapping of *Rpv8*, a locus conferring resistance to grapevine downy mildew. *Theor. Appl. Genet.* 123: 43-53

Brewer MT and Milgroom MG (2010) Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator*, from diverse Vitis species. *BMC Evol. Biol.* 10: 268.

Cadle-Davidson L (2008) Variation within and between *Vitis* spp. for foliar resistance to the downy mildew pathogen *Plasmopora viticola*. *Plant Disease* 92: 1577-84

Consonni C, Humphry ME, Hartmann HA, Livaja M, Durner J, Westphal L, Vogel J, Lipka V, Kemmerling B, Schulze-Lefert P, Somerville SC and Panstruga R (2006) Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. *Nature Genetics* 38: 716-720

Godfrey D, Bohlenius H, Pedersen C, Zhang Z, Emmersen J and Thordal-Christensen H (2010) Powdery mildew fungal effector candidates share N-terminal Y/F/WxC-motif. *BMC Genomics* 11:317

De Block M, Debrouwer D (1991) Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. *Theor. Appl. Genet.* 82: 257-63.

Derozier S, Samson F, Tamby JP, Guichard C, Brunaud V, Grevet P, Gagnot S, Label P, Leple JC, Lecharny A and Aubourg S. (2011) Exploration of plant genomes in the FLAGdb(++) environment. *Plant Methods* 7: 8.

Deslandes L and Rivas S (2011) The plant cell nucleus: a true arena for the fight between plants and pathogens. *Plant Signal Behav.* 6: 42-48.

Dinesh-Kumar SP and Baker BJ (2000) Alternatively spliced N resistance gene transcripts: Their possible role in tobacco mosaic virus resistance. *Proc. Natl. Acad. Sci. USA* 97: 1908-1913.

Donald TM, Pellerone F, Adam-Blondon AF, Bouquet A, Thomas MR and Dry IB (2002) Identification of resistance gene analogs linked to a powdery mildew resistance locus in grapevine. *Theor. Appl. Genet.* 104: 610-618.

Eibach R, Zyprian E, Welter L and Topfer R (2007) The use of molecular markers for pyramiding resistance genes in grapevine breeding. *Vitis* 46: 120-124.

Ellis JG, Dodds PN and Lawrence GJ (2007) Flax rust resistance gene specificity is based on direct resistance-avirulence protein interactions. *Annu. Rev. Phytopathol.* 45: 289-306.

Feechan A, Jermakow AM, Torregrosa L, Panstruga R and Dry IB (2008) Identification of grapevine *MLO* gene candidates involved in susceptibility to powdery mildew. *Funct. Plant Biol.* 35: 1255-1266.

Feechan A, Jermakow AM, Ivancevic A, Godfrey D, Pak H, Panstruga R, Dry IB (2013) Host cell entry of powdery mildew is correlated with endosomal transport of antagonistically acting VvPEN1 and VvMLO to the papilla. *Molecular Plant Microbe Interactions* 26: 1138-1150.

Feechan A, Anderson C, Torregrosa L, Jermakow A, Mestre P, Wiedemann-Merdinoglu S, Merdinoglu D, Walker AR, Cadle-Davidson L, Reisch B, Aubourg S, Bentahar N, Shrestha B, Bouquet A, Adam-Blondon A-F, Thomas MR, Dry IB (2013) Genetic dissection of a TIR-NB-LRR locus from the wild North American grapevine species *Muscadinia rotundifolia* identifies paralogous genes conferring resistance to major fungal and oomycete pathogens in cultivated grapevine. *Plant J.* 76: 661-674.

Ficke A, Gadoury DM, Seem RC and Dry IB (2003) Effects of ontogenic resistance upon establishment and growth of *Uncinula necator* on grape berries. *Phytopathology* 93: 556-563

Gassmann W (2008) Alternative splicing in plant defense. *Curr. Top. Microbiol. Immunol.* 326: 219-233.

Humphry M, Reinstädler A, Ivanov S, Bisseling T and Panstruga R (2011) Durable broad-spectrum powdery mildew resistance in pea er1 plants is conferred by natural loss-of-function mutations in PsMLO1. *Mol. Plant Pathol.* 12: 866-78.

Iocco P, Franks T and Thomas MR (2001) Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. *Transgenic Res.* 10: 105-112.

Jaillon, O., Aury, J.M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyere, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Del Fabbro, C., Alaux, M., Di Gaspero, G., Dumas, V., Felice, N., Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Le Clainche, I., Malacrida, G., Durand, E., Pesole, G., Laucou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pe, M.E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A.F., Weissenbach, J., Quetier, F. and Wincker, P. (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature*, 449, 463-467.

Koch E and Slusarenko A (1990) *Arabidopsis* is susceptible to infection by a downy mildew fungus. *Plant Cell* 2: 437-445.

Leach, J.E., Cruz, V., Casiana, M., Bai, J. and Leung, H. (2001) Pathogen fitness penalty as a predictor of durability of disease resistance genes. Annual Review of Phytopathology 39, 187–224.

McKnight TD, Lillis MT and Simpson RB (1987) Segregation of genes transferred to one plant cell from two separate Agrobacterium strains. *Plant Mol. Biol.* 8: 439-445.

Meyers, B.C., Shen, K.A., Rohani, P., Gaut, B.S. and Michelmore, R.W. (1998) Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. *Plant Cell* 10: 1833-1846.

Miller M, Tagliani L, Wang N, Berka B, Bidney D and Zhao ZY (2002) High efficiency transgene segregation in co-transformed maize plants using an *Agrobacterium tumefaciens* T-DNA binary system. *Transgenic Res.* 11: 381-396.

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M. and Narusaka, Y. (2009) *RRS1* and *RPS4* provide a dual resistance-gene system against fungal and bacterial pathogens. *Plant J*. 60: 218-226.

Parra G, Bradnam K, Rose AB and Korf I (2011) Comparative and functional analysis of intron-mediated enhancement signals reveals conserved features among plants. *Nucleic Acids Res.* 39: 5328-37.

Pedersen C, Ver Loren van Themaat E, McGuffin LJ, Abbott JC, Burgis TA, Barton G, Bindschedler LV, Lu X, Maekawa T, Wessling R, Cramer R, Thordal-Christensen H, Panstruga R and Spanu PD (2012) Structure and evolution of barley powdery mildew effector candidates. *BMC Genomics* 13: 694.

Peressotti, E., Wiedemann-Merdinoglu, S., Delmotte, F., Bellin, D., Di Gaspero, G., Testolin, R., Merdinoglu, D. and Mestre, P. (2010) Breakdown of resistance to grapevine downy mildew upon limited deployment of a resistant variety. *BMC Plant Biol.* 10: 147.

Ramming DW, Gabler F, Smilanick J, Cadle-Davidson M, Barba P, Mahanil S and Cadle-Davidson L (2011) A single dominant locus, *Ren4*, confers rapid non-race-specific resistance to grapevine powdery mildew. *Phytopathology* 101: 502-508.

Schiex, T., Moisan, A. and Rouze, P. (2001) Eugene: An eukaryotic gene finder that combines several sources of evidence. In *Computational Biology* (Gasceul, O. and Sagot, M-F., eds). Springer-Verlag, pp. 111-125.

Schofield P, Morison J (2010) Assessment of Economic Cost of Endemic Pests & Diseases on the Australian Grape & Wine Industry" Final Report.

Seifi A, Kaloshian I, Vossen J, Che DD, Bhattarai KK, Fan JM, Naher Z, Goverse A, Tjallingii WF, Lindhout P, Visser RGF and Bai YL (2011) Linked, if not the same, *Mi-1* homologues confer resistance to tomato powdery mildew and root-knot nematodes. *Mol. Plant Microbe Interact.* 24: 441-450.

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW and Rezaian MA (2005) A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. *Plant Cell* 17: 311-325.

Shen QH, Saijo Y, Mauch S, Biskup C, Bieri S, Keller B, Seki H, Ulker B, Somssich IE and Schulze-Lefert, P (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* 315: 1098-1103.

Steffenson BJ (1992) Analysis of durable resistance to stem rust in barley. *Euphytica* 63: 153-167.

Takken FLW, Albrecht M and Tameling WIL (2006) Resistance proteins: molecular switches of plant defence. *Curr. Opinion in Plant Biol.* 9: 383-390.

van der Vossen EA, van der Voort JN, Kanyuka K, Bendahmane A, Sandbrink H, Baulcombe DC, Bakker J, Stiekema WJ and Klein-Lankhorst RM (2000) Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. *Plant J*. 23: 567-576.

Wan Y, Scwaninger H, He P and Wang Y (2007) Comparison of resistance to powdery mildew and downy mildew in Chinese wild grapes. *Vitis* 46: 132-136.

Wang W, Wen Y, Berkey R and Xiao S (2009) Specific targeting of the Arabidopsis resistance protein RPW8.2 to the interfacial membrane encasing the fungal Haustorium renders broad-spectrum resistance to powdery mildew. *Plant Cell* 21: 2898-2913.

Wang Y, Liu Y, He P, Chen O, Lamikanra O and Lu J (1995) Evaluation of foliar resistance to *Uncinula necator* in Chinese wild Vitis species. *Vitis* 34: 159-164.

Zhang XC and Gassmann W (2003) RPS4-mediated disease resistance requires the combined presence of *RPS4* transcripts with full-length and truncated open reading frames. *Plant Cell* 15: 2333-2342.

Zheng Z, Nonomura T, Appiano M, Pavan S, Matsuda Y, Toyoda H, Wolters AM, Visser RG and Bai Y (2013) Loss of function in Mlo orthologs reduces susceptibility of pepper and tomato to powdery mildew disease caused by *Leveillula taurica*. PLoS ONE 8: e70723.

Zhou F, Kurth J, Wei F, Elliott C, Vale G, Yahiaoui N, Keller B, Somerville S, Wise R and Schulze-Lefert, P (2001) Cell-autonomous expression of barley *Mla1* confers race-specific resistance to the powdery mildew fungus via a *Rar1*-independent signaling pathway. *Plant Cell* 13: 337-350.

Appendix 4: Staff

Project Staff

Dr Ian Dry	CSIRO Plant Industry, Adelaide
Dr Mark Thomas	CSIRO Plant Industry, Adelaide
Dr Angela Feechan, Postdoctoral Fellow (1/07/09 - 29/3/13)	CSIRO Plant Industry, Adelaide
Ange Jermakow, Technical Officer	CSIRO Plant Industry, Adelaide
Nicole Kempster, Technical assistant (1/07/09 - 14/02/12)	CSIRO Plant Industry, Adelaide
Debra McDavid, Technical Officer (30%; 1/07/09 - 30/06/2010) (100%; 1/07/2012 - 30/0613)	CSIRO Plant Industry, Adelaide
Nayana Arunasiri, Technical Officer - (30/4/12 onwards)	CSIRO Plant Industry, Adelaide

Collaborators

Dr Anne-Francoise Adam & Dr Sebastien Aubourg - INRA-URGV, Evry, France.

Dr Laurent Torregrosa & Dr Bipna Shresta - ENSA-M/INRA, UFR de Viticulture, Montpellier, France.

Dr Père Mestre - INRA, UMR1131 Sante Vigne & Qualite Vin, 68000 Colmar, France.

Dr Lance Cadle-Davidson - USDA-ARS Grape Genetics Res. Unit, Geneva, NY 14456, USA.

Dr Bruce Reisch - Horticulture Dept, New York Agricultural Station, Cornell University, Geneva, NY 14456, USA

Dr David Ramming - USDA/ARS/CDPG , Parlier, California, 93648, USA.

Dr Ralph Panstruga - Institute for Biology, Unit of Plant Molecular Cell Biology, Worringerweg 1, 52056 Aachen, Germany

This page intentionally left blank

Appendix 5: Supplementary data

Table 5. Nucleotide sequences of oligonucleotides used in this study

Primer name	Oligonucleotide sequence
AJ102	5'-ACCTCTTTCGAATATTATCACGA
AJ103	5'-GATATGTGCTGACCCACAC
CB20	5'-GGTAGGAGAGGGATTCGCACC
CB319	5'-CGATTGCTCCAGAACTCTTG
CB563	5'-CCATCATGTGATTATAGTTCTGC
CB568	5'-GTCACCAAGTGACAAATTCCTG
CB600	5'-CAGCGCATGGAGATAGTG
CB631	5'-TGTTGAACTTCACTTGAAGTGTAGC
CB632	5'-GCTACACTTCAAGTGAAGTTCAACA
EF1F	5'-CGGGCAAGAGATACCTCAAT
EF1R	5'-AGAGCCTCTCCCTCAAAAGG
GLP1-12P1	5'-GGAATATTTACTTGGACATCG
GLP1-12P3	5'-CATTTGAATTGGAGCATACTC
Oligo-dt adapter	5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT
RGA1F	5'-CATTGGGAAGGGTAGCAAG
RGA1R	5'-GTGGAATTTATCTGCACGATG
RGA2F	5'-CAGTTTATTTCCGCCATGCTG
RGA2R	5'-GCAATAACACCAGGATGAATGAA
RGA4F	5'-CATCTTATGATACCGCAACTC
RGA4R	5'-CAATTACACCAGCATGAATGAG
RGA8F	5'-CATGTGTTTATCGTCCATCATG
RGA8R	5'-CACCAGCATTCATGATAGCATA
RGA8-ATGF	5'-GAACTCGAGATGGCTTCCACAAGCTCCTTCAGA
RGA8-KPNR	5'-GAAGGTACCCAAGCGAGATCTCGGTGTGGGG
RGA8-NLS-SDMF	5'-GAAGACACTCTTGGAAACCGTACTAGTAGACGTAATGATTCCCTAC
RGA8-NLS-SDMR	5'-GTAGGGAATCATTACGTCTACTAGTACGGTTTCCAAGAGTGTCTTC
RGA8 3'*Sbf*I/*Kpn*I-R	5'- CTAGGTACCATCCTGCAGGGTGATACAGACCTTTGTCAC
RGA9F	5'-CGTGCAGATGCTCGTGGC
RGA9R	5'-CGCATACTACATCTCTCGGATA
RGA10F	5'-CCTGAAGCGGAAATTCTCAG
RGA10R	5'-TGCATGGAAATCACAAGCATCT
RGA10-ATGF	5'-GACCTCGAGATGGCTTCCACAAGCTCC
RGA10 B/C-F	ACTGATAGCATATTCCGTCGAT
RGA10 3'*Sma*I/*Kpn*I-R	GATCCCGGGATCCTGCAGGGCTCTTGAGTAATAAACCCC
RGA10-XBAR	5'-GAATCTAGACCACATGTGATTTTGTTCTGATTG
RGA10-NLS-SDMF	5'-GAAGACACTCTTGGAAACCGTACTAGTAGACGTGATGATTCCCTAC
RGA10-NLS-SDMR	5'-GTAGGGAATCATCACGTCTACTAGTACGGTTTCCAAGAGTGTCTTC
RGA11F	5'-GACCTGATTATGATCCTGCATG
RGA11R	5'-CACTCACACGAGCGTGAAG
VvMLO3-hp1-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATAGAGTGATGAAGATTGGATTG
VvMLO3-hp1-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGAAAGCATTGGCTTGATTTTGG
VvMLO4-hp1-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATCCCCGAGAAGGTCCAAC
VvMLO4-hp1-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGGAAAATTCAATCTCCACGG
VvMLO13-hp1-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGATGAACTTGAAGATC
VvMLO13-hp1-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGGCCTTGGGCTCCTTGGGC
VvMLO17-hp1-F	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAAAAGGAGAAATTTGAATCCAG
VvMLO17-hp1-R	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAAGAAAAGTATATTGAAATCACAAG

This page intentionally left blank

Appendix 6: Budget reconciliation

An End of Project Financial statement will be submitted via the GWRDC's Clarity Investment Management System.